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(71) Applicant (for all designated States except US): CALGENE, INC. [US/US]; 1920 Fifth Street, Davis, CA 95616 (US).

## (72) Inventors; and

(75) Inventors/Applicants (for US only) : DAVIES, Huw, Maelor [GB/US]; 307 Grande Avenue, Davis, CA 95616 (US). POLLARD, Michael, Roman [GB/US]; 5649 East Buckeye Road, Madison, WI 53716 (US). VOELKER, Toni, Alois [DE/US]; 1206 Covell Place, Davis, CA 95616 (US). THOMPSON, Gregory, A. [US/US]; 5127 Cowel Blvd., Davis, CA 95616 (US).

(74) Agents: LASSEN, Elizabeth et al.; Calgene, Inc., 1920 Fifth Street, Davis, CA 95616 (US).

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(54) Title: PLANT THIOESTERASES

## (57) Abstract

This invention relates to plant thioesterases, means to identify such proteins, amino acid and nucleic acid sequences associated with such protein, methods to obtain, make and/or use such plant thioesterases. Also, by this invention, the existence of a heretofore unproven factor critical to the biosynthesis of medium-chain fatty acids in plants is demonstrated.

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## PLANT THIOESTERASES

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This application is a continuation-in-part of USSN 07/662,007 filed February 27, 1991, and a continuation-in-part of USSN 07/514,030 filed April 26, 1990 and a continuation-in-part of USSN 07/620,426 filed November 30, 10 1990.

### Technical Field

The present invention is directed to protein preparations, amino acid and nucleic acid sequences and 15 constructs, and methods related thereto.

### **INTRODUCTION**

#### Background

"Improved" means to obtain or manipulate fatty acid 20 compositions, from biosynthetic or natural plant sources, are needed. For example, novel oil products, improved sources of synthetic triacylglycerols (triglycerides), alternative sources of commercial oils, especially tropical oils (i.e., palm kernel and coconut oils), and plant oils 25 found in trace amounts from natural sources are desired for a variety of industrial and food uses.

To this end, the Fatty Acid Synthesis (FAS) system in plants and bacteria, FAS-II, has been studied. The mechanism of producing "long-chain fatty acids", i.e., 30 fatty acids having a carbon chain length of equal to or greater than 16 carbon atoms (C16), via the acyl carrier

protein (ACP)-dependent, plastid-localized FAS system of plants is relatively well characterized. However, the amino acid and corresponding nucleic acid sequences of many of the proteins responsible for this activity have not been

5 determined. In particular, the enzyme by which free long-chain fatty acids are produced has been studied in several different crops. Nevertheless, the mechanism(s) by which plants produce fatty acids having shorter carbon chains, i.e., less than C16 atoms, including short-chain free fatty

10 acidy (C4-C8) and medium-chain free fatty acids (C8-C14), has remained elusive.

Characterization of thioesterases (also known as hydrolases) would be useful for the further study of plant FAS systems and for the development of novel and/or

15 alternative oils sources. Generating a spread of C4, C6 and C8 short chain 3-keto fatty acids could become a key improvement in polyhydroxybutyrate (PHB)-based biodegradable plastics made in bacteria and plants.

Medium-chain fatty acids have special importance in the

20 detergent and lubricant industries or in the formulation of edible oils with reduced caloric value or other health benefits. See for example, U.S. Pat. No. 4,863,753 and Barch, A.C. & Babayan, V.K., Am. J. Clin. Nat. (1982) 36:950-962. Longer chain fatty acids may have certain

25 other utilities, i.e., C16 and C18 have particular uses in margarine and other solid oil-based products and very long chain fatty acids also have specialized uses, i.e., C22 is used to make peanut butter smoother. As such, a ready source of a variety of fatty acid lengths, including

storage lipids which have incorporated differing chain length fatty acids in desired ratios, are desired for a variety of industrial and food use fields. As the biosynthetic pathway for chain termination of fatty acids 5 in plants is determined, the system can be adapted for application *in vivo* and *in vitro*.

Thus, studies of plant chain termination mechanisms may provide means to further enhance, control, modify or otherwise alter the length of fatty acid products or 10 resulting triglycerides and oils. And, the elucidation of the factor(s) critical to the natural production of free fatty acids in plants is desired, including the purification of such factors and the characterization of element(s) and/or co-factors which enhance the efficiency 15 of the system. Of special interest are the nucleic acid sequences of genes encoding factors related to the production of such free fatty acids for applications in genetic engineering.

20 Relevant Literature

McKeon, T.A. & Stumpf, P.K., *J. Biol. Chem.* (1982) 257:12141-12147 reports a 700-fold purification of safflower acyl ACP-thioesterase. Other references reporting the purification and characterization of long-chain acyl-ACP thioesterases include Shire, et al., *Arch. Biochem. Biophys.* (1976) 172:110-116; Ohlrooge, et al., *Arch. Biochem Biophys.* (1978) 189:382-391; Imai, et al., *Plant Lipid Biochemistry, The Ninth International Symposium on Plant Lipids, Wye College, Univ. of London, July 8-13*

(1990); Hellyer, A. & Slabas, A.R., *Plant Lipid Biochemistry*, The Ninth International Symposium on Plant Lipids, Wye College, Univ. of London, July 8-13 (1990).

- P.K.Stumpf, *The Biochemistry of Plants* (P.K. Stumpf & E. Conn, eds.) (1987) 9:121-136, summarizes mechanisms of termination of the fatty acid chain elongation pathway of a variety of chain-lengths in plants. Specific thioesterases for producing medium-chain fatty acids are postulated as well as other possible explanations. Harwood, J.L., *Ann. Rev. Plant Physiol. Mol. Bio.* (1988) 39:101-138, references various possibilities in the literature regarding production of large amounts of medium-chain length fatty acids in some plant tissues and reports that all attempts to find a "suitable thioesterase" responsible for medium-chain fatty acid production have proved negative. Harwood, J.L., *Crit. Rev. Plant Sci.* (1989) 8:1-43, reviews current information regarding the production of medium-chain fatty acids in plants with the conclusion that very little is known. See also, Pollard, M.R. and Singh, S.S., *The Metabolism, Structure and Function of Plant Lipids*, Stumpf, P.K., Mudd, J.B., and Nes, W.D., eds. (Plenum Press, NY 1987) pp. 455-463.

#### BRIEF DESCRIPTION OF THE FIGURES

- Figure 1. Two peptide sequences and the degenerate oligonucleotides used in the PCR reaction to obtain the Bay thioesterase sequence are shown. "I" in the oligonucleotide sequences represents the nucleotide inosine. The lower case DNA sequence represent artificial

5' ends designed to allow for subsequent cloning with the two chosen restriction enzymes (restriction sites underlined). The oligonucleotide for peptide 701 (SEQ ID NO: 14) is SEQ ID NO: 32 and for peptide 698 (SEQ ID NO: 12) is SEQ ID NO: 33.

Figure 2. A fusion of both the PCR generated cDNA and the longest library clone of the Bay thioesterase is shown. The first 210 bases (SEQ ID NO: 34) are from the 800 bp PCR product. The gap represents unsequenced DNA, about 240 bp, as determined by restriction enzyme mapping. The remaining sequence (SEQ ID NO: 35) is from the PCR fragment and the library clone. Translation into the proper frame is shown under the sequence. Selected peptide sequences are depicted by horizontal lines under the respective protein sequence. Numbers shown correspond to those provided in Table 8. Mismatches with the sequence provided through protein sequencing are shown.

Figure 3. A sequence comparison is shown between two related Bay thioesterase cDNA clones isolated using the 800 bp PCR-generated fragment described in Example 14.C.2. Sequence identity is shown by horizontal lines.

Figure 4. A full length sequence of a Bay thioesterase is shown. In Figure 4A, the amino acid sequence of the structural gene is given. In Figure 4B, the nucleic acid sequence is given. The amino acid sequence in Figure 4A begins with the ATG codon at 181 to 183. As noted elsewhere in the specification, three possible ATG start codons are located in the first 219 base pairs of the nucleic acid sequence of Figure 4B.

Figure 5. A representation of the orientation of the fragments shown in Table 9 is provided.

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## SUMMARY OF THE INVENTION

This invention relates to plant thioesterases and encompasses both shorter-chain preferring and longer-chain preferring acyl-carrier substrate thioesterases. Especially of interest are conserved amino acid or nucleic acid sequences between such shorter-chain preferring and such longer-chain preferring acyl-carrier thioesterases. Methods are used such conserved sequences to obtain plant thioesterases is described.

In a first embodiment, this invention is directed to nucleic acid sequences which encode a plant thioesterase. This includes sequences which encode biologically active plant thioesterases as well as sequences which are to be used as probes, vectors for transformation or cloning intermediates. Biologically active sequences may be found in a sense or anti-sense orientation as to transcriptional regulatory regions found in various constructs. The plant thioesterase encoding sequence may encode a complete or partial sequence depending upon the intended use. All or a portion of the genomic sequence, cDNA sequence, precursor plant thioesterase or mature plant thioesterase is intended.

Of special interest are recombinant DNA constructs which can provide for the transcription or transcription and translation (expression) of the plant thioesterase

sequence. In particular, constructs which are capable of transcription or transcription and translation in plant host cells are preferred. Such construct may contain a variety of regulatory regions including transcriptional initiation regions obtained from genes preferentially expressed in plant seed tissue.

In a second aspect, this invention relates to the presence of such constructs in host cells, especially plant host cells.

10 In a different aspect, this invention relates to transgenic host cells which have an expressed plant thioesterase therein.

In yet a different aspect, this invention relates to a method for producing a plant thioesterase in a host cell or progeny thereof via the expression of a construct in the cell. Cells containing a plant thioesterase as a result of the production of the plant thioesterase encoding sequence are also contemplated herein.

20 In a different embodiment, this invention relates to methods of using a DNA sequence encoding a plant thioesterase for the modification of the proportion of free fatty acids produced within a cell, especially plant cells. Plant cells having such a modified free fatty acid composition are also contemplated herein.

25 In a further aspect of this invention, medium-chain preferring plant thioesterase proteins and sequences which are related thereto, including amino acid and nucleic acid sequences, are contemplated. Medium-chain preferring acyl-carrier thioesterases substantially free of other plant

proteins are described. Medium-chain preferring acyl-carrier thioesterases which demonstrate preferential hydrolysis activity toward acyl-acyl carrier protein (ACP) substrates are of particular interest. Nucleic acid sequences and amino acid sequences of such proteins are 5 described.

In addition, methods to produce a medium-chain free fatty acid utilizing a medium-chain preferring acyl-carrier thioesterase is provided.

10 Plant thioesterases exemplified herein include an *Umbellularia californica* (Bay), *Cuphea hookeriana* (*Cuphea*) and *Carthamus tinctorius* (safflower) thioesterases. These exemplified thioesterases may be used to obtain other plant thioesterases of this invention.

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#### DETAILED DESCRIPTION OF THE INVENTION

A plant thioesterase of this invention includes any sequence of amino acids, such as a protein, polypeptide or peptide fragment obtainable from a plant source which 20 demonstrates the ability to catalyze the production of free fatty acid(s) from fatty acyl-carrier substrates under plant enzyme reactive conditions. By "enzyme reactive conditions" is meant that any necessary conditions are available in an environment (i.e., such factors as 25 temperature, pH, lack of inhibiting substances) which will permit the enzyme to function.

Preferential activity of a plant thioesterase toward a particular chain-length fatty acyl-carrier substrate is determined upon comparison of free fatty acid product

amounts obtained per different chain length substrates. For example, by "C12 preferring" is meant that the hydrolytic activity of the enzyme preparation demonstrates a preference for lauroyl, and perhaps decanoyl, over other 5 substrates of different acyl carbon lengths. In a like fashion, a plant thioesterase having "C10 preferring" activity will show higher levels of activity toward decanoyl substrates, and perhaps octanoyl, over other substrates of different acyl carbon lengths. It is noted 10 that some activity, of a significantly lesser magnitude, may be observed against other chain-length fatty acyl substrates, i.e., the specificity will be substantial, but may not be absolute.

As noted above, a plant thioesterase of this invention 15 will display activity toward fatty acyl-carrier substrates. During biosynthesis of lipids in a plant cell, fatty acids are typically covalently bound to ACP or coenzyme A (CoA) carriers. Plant thioesterases which display preferential activity toward acyl-ACP substrates are especially 20 preferred because they are likely to be closely associated with the FAS pathway in immature embryo plastids. However, activity toward acyl-CoA substrates or other synthetic substrates, for example, is also contemplated herein.

Other plant thioesterases are obtainable from the 25 specific exemplified protein preparations and sequences provided herein. Furthermore, it will be apparent that one can obtain natural and synthetic plant thioesterases, including modified amino acid sequences and starting materials for synthetic-protein modeling from the

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exemplified plant thioesterases and from plant thioesterases which are obtained through the use of such exemplified sequences. Modified amino acid sequences include sequences which have been mutated, truncated, 5 increased and the like, whether such sequences were partially or wholly synthesized. Sequences which are actually purified from plant preparations or are identical or encode identical proteins thereto, regardless of the method used to obtain the protein or sequence, are equally 10 considered naturally derived.

Thus, one skilled in the art will readily recognize that antibody preparations, nucleic acid probes (DNA and RNA) and the like may be prepared and used to screen and recover "homologous" or "related" thioesterases from a 15 variety of plant sources. Typically, nucleic acid probes are labeled to allow detection, preferably with radioactivity although enzymes or other methods may also be used. For immunological screening methods, antibody preparations either monoclonal or polyclonal are utilized. 20 Polyclonal antibodies, although less specific, typically are more useful in gene isolation. For detection, the antibody is labeled using radioactivity or any one of a variety of second antibody/enzyme conjugate systems that are commercially available. Examples of some of the 25 available antibody detection systems are described by Oberfielder (*Focus* (1989) BRL Life Technologies, Inc., 11:1-5).

Homologous sequences are found when there is an identity of sequence and may be determined upon comparison

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of sequence information, nucleic acid or amino acid, or through hybridization reactions between a known thioesterase and a candidate source. Conservative changes, such as Glu/Asp, Val/Ile, Ser/Thr, Arg/Lys and Gln/Asn may 5 also be considered in determining sequence homology. Typically, a lengthy nucleic acid sequence may show as little as 50-60% sequence identity, and more preferably at least about 70% sequence identity, between the target sequence and the given plant thioesterase of interest 10 excluding any deletions which may be present, and still be considered related. Amino acid sequences are considered homologous by as little as 25% sequence identity between the two complete mature proteins. (See generally, Doolittle, R.F., *OF URFS and ORFS* (University Science Books, CA, 1986.)

A genomic or other appropriate library prepared from the candidate plant source of interest may be probed with conserved sequences from the plant thioesterase to identify homologously related sequences. Use of an entire cDNA or 20 other sequence may be employed if shorter probe sequences are not identified. Positive clones are then analyzed by restriction enzyme digestion and/or sequencing. When a genomic library is used, one or more sequences may be identified providing both the coding region, as well as the 25 transcriptional regulatory elements of the thioesterase gene from such plant source. Probes can also be considerably shorter than the entire sequence.

Oligonucleotides may be used, for example, but should be at least about 10, preferably at least about 15, more

preferably at least 20 nucleotides in length. When shorter length regions are used for comparison, a higher degree of sequence identity is required than for longer sequences. Shorter probes are often particularly useful for polymerase 5 chain reactions (PCR), especially when highly conserved sequences can be identified. (See, Gould, et al., PNAS USA (1989) 86:1934-1938.)

When longer nucleic acid fragments are employed (>100 bp) as probes, especially when using complete or large cDNA 10 sequences, one would screen with low stringencies (for example 40-50°C below the melting temperature of the probe) in order to obtain signal from the target sample with 20-50% deviation, i.e., homologous sequences. (See, Beltz, et al. *Methods in Enzymology* (1983) 100:266-285.).

15 In a preferred embodiment, a plant thioesterase of this invention will have at least about 30% sequence identity, and more preferable at least about 50% sequence identity with at least a sequence of 8 amino acids of an exemplified plant thioesterase or a plant thioesterase 20 which has in turn been obtained from a plant thioesterase sequence. Alternatively, a plant thioesterase of this invention will have at least about 65% sequence identity and more preferably at least about 75% sequence homology with an exemplified plant thioesterase or a plant 25 thioesterase which in turn has been obtained from a given plant thioesterase sequence. In particular, thioesterases which are obtainable from an amino acid or nucleic acid sequence of a Bay thioesterase (See, Fig. 4A or B) or a safflower amino acid fragment of Table 9 (Example 14,

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*infra)* are especially preferred. The plant thioesterase may have preferential activity toward longer or shorter chain fatty acyl substrates. Plant thioesterases having long-chain preferring fatty acyl hydrolysis activity or 5 medium-chain preferring fatty acyl hydrolysis activity are both considered homologously related proteins hereunder, for reasons as described in more detail further below.

Again, not only can sequences such as shown in Fig. 4 and Table 9 be used to identify homologous plant 10 thioesterases, but the resulting sequences obtained therefrom may also provide a further method to obtain plant thioesterases from other plant sources. In particular, PCR may be a useful technique to obtain related plant thioesterases from sequence data provided herein. One 15 skilled in the art will be able to design oligonucleotide probes based upon sequence comparisons or regions of typically highly conserved sequence. Of special interest are probes based upon the S828 or the S829 fragment of Table 9. Details relating to the design and methods for a 20 PCR reaction using these probes is described more fully in the examples.

It should also be noted that plant thioesterases of a variety of sources can be used to investigate chain termination events of plant fatty acid biosynthesis in a 25 wide variety of plant and *in vivo* applications. Because all plants appear to synthesize fatty acids via a common metabolic pathway, the study and/or application of one plant thioesterase to a heterologous plant host may be readily achieved in a variety of species. In other

applications, a plant thioesterase can be used in conjunction with plastid lysates outside the native plant source of the thioesterase to enhance the production and/or modify the composition of the fatty acids prepared in 5 *vitro*.

Once the nucleic acid sequence is obtained, the transcription, or transcription and translation (expression), of the plant thioesterase in a host cell is desired to produce a ready source of the enzyme and/or 10 modify the composition of fatty acids and/or triglycerides found therein. Other useful applications may be found when the host cell is a plant host cell, *in vitro* and *in vivo*.

For example, by increasing the amount of a respective shorter-chain preferring thioesterase available to the 15 plant FAS complex, an increased percentage of shorter chain fatty acids may be provided. In a like manner, for some applications it may be desired to decrease the amount of shorter-chain preferring acyl-ACP thioesterase endogenously expressed in a plant cell by anti-sense technology, for 20 example, to increase the percentage of longer chain fatty acids, and visa versa. See, co-pending U.S. patent application No. 240,408 filed 8/30/88. The greater specificity of the plant thioesterase toward a given fatty-acyl substrate, the more control it may be possible to 25 exert in the FAS system.

#### Medium-Chain Preferring Plant Thioesterases

By this invention, a mechanism for the biosynthesis of medium-chain fatty acids in plants is demonstrated. Namely, that specific plant thioesterases having

preferential activity toward medium-chain acyl substrates are involved in the accumulation of medium chain fatty acids in at least some plant species.

The determination that chain-length specific plant thioesterases are active in the in vivo production of medium-chain fatty acids suggests several possibilities for enzyme plant sources. And in fact, medium-chain fatty acids are found in some natural plant species in abundance. For example, several species in the genus *Cuphea* accumulate triglycerides containing medium-chain fatty acids in their seeds, e.g., *procumbens*, *lutea*, *hookeriana*, *hyssopifolia*, *wrightii* and *inflata*. Another natural plant source of medium-chain fatty acids are seeds of the Lauraceae family: e.g., the California Bay (*Umbellularia californica*), *Pisa* (*Actinodophne hookeri*), Sweet Bay (*Laurus nobilis*) and *Cinnamomum camphora* (camphor). Other plant sources include *Ulmaceae* (elm), *Myristicaceae*, *Simarubaceae*, *Vochysiaceae*, and *Salvadoraceae*.

Plants having significant presence of medium-chain fatty acids therein are preferred candidates to obtain naturally-derived medium-chain preferring plant thioesterases. However, it will also be recognized that other plant sources which do not have a significant presence of medium-chain fatty acids may be readily screened as other enzyme sources. In addition, a comparison between endogenous medium-chain preferring plant thioesterases and between longer and/or shorter chain preferring plant thioesterases may yield insights for protein modeling or other modifications to create synthetic

medium-chain preferring plant thioesterases as discussed above.

Especially of interest are medium-chain preferring plant thioesterases which demonstrate preferential hydrolysis activity toward fatty acyl-ACP substrates. Most preferred are medium-chain preferring plant thioesterases which demonstrate a marked preference toward C12 acyl-ACP, C10 acyl-ACP or C8 acyl-ACP substrates. As described above, other plant sources may also provide sources for these enzymes through the use of protein purification, nucleic acid probes, antibody preparations, protein modeling, or sequence comparisons, for example, and of special interest are the respective amino acid and nucleic acid sequences corresponding to such plant thioesterases.

Also as previously described, once nucleic acid sequence is obtained for the given plant thioesterase, further plant sequences may be compared and/or probed to obtain homologously related DNA sequences thereto and so on.

Medium-chain preferring acyl-ACP plant thioesterases have been partially purified from immature embryos of the California Bay (*Umbellularia californica*) tree, hereinafter sometimes referred to as "Bay," and *Cuphea hookeriana*, hereinafter sometimes referred to as "Cuphea." The Bay thioesterase enzyme activity consistently co-migrates in chromatographic and electrophoretic separations with a protein or pair of proteins having an apparent Mr of approximately 34 kD. A native molecular weight of approximately 42 kD has been estimated by gel filtration chromatography suggesting that the enzyme is a monomer of

medium-chain preferring plant thioesterases as discussed above.

- Especially of interest are medium-chain preferring plant thioesterases which demonstrate preferential hydrolysis activity toward fatty acyl-ACP substrates. Most preferred are medium-chain preferring plant thioesterases which demonstrate a marked preference toward C12 acyl-ACP, C10 acyl-ACP or C8 acyl-ACP substrates. As described above, other plant sources may also provide sources for these enzymes through the use of protein purification, nucleic acid probes, antibody preparations, protein modeling, or sequence comparisons, for example, and of special interest are the respective amino acid and nucleic acid sequences corresponding to such plant thioesterases.
- Also as previously described, once nucleic acid sequence is obtained for the given plant thioesterase, further plant sequences may be compared and/or probed to obtain homologously related DNA sequences thereto and so on.

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the 34 kD subunit. Affinity chromatography on immobilized ACP forms a critical step in the purification procedure, and resolves the 12:0-ACP and 18:1-ACP thioesterases sufficiently to confirm that the medium-chain enzyme has  
5 negligible action on 18:1-ACP. The time-course of induction of 12:0-ACP thioesterase during seed development shows that the fatty acyl composition of the cotyledons changes abruptly from long-chain acyl groups to predominantly C10 and C12 at the earliest appearance of  
10 enzyme activity.

As demonstrated more fully in the Examples, a plant thioesterase preparation having preferential hydrolase activity toward medium-chain fatty acyl-ACP substrates of California Bay substantially free of other plant proteins  
15 is obtainable as follows: Briefly, a supernatant fraction of ground California Bay immature embryos is subjected to ammonium sulfate fractionation, followed by hydroxyapatite column chromatography of the redissolved pellet, applying carboxymethyl Sepharose chromatography to the eluted fractions, and further chromatography on a column of immobilized *E. coli* ACP. One or two proteins having an approximate molecular weight of 34 kD co-elutes or co-migrate with the enzyme activity through a variety of chromatographic or electrophoretic techniques. These  
20 proteins correspond to the medium-chain thioesterase. (See also, Pollard et al., *Archiv. Biochem Biophys.* (1991) 284:306-312, which is hereby incorporated by reference.)

Also described in the Examples, are methods to obtain a partially purified *Cuphea* C10-preferring acyl-ACP

thioesterase. The *Cuphea* thioesterase is partially purified from other plant proteins and activity is confirmed in the same general manner as the Bay thioesterase. As described more fully in the Examples, the 5 various buffers and techniques may be different than those used in the Bay extraction. Enzyme activity is compared against various acyl-ACP substrates and demonstrates significantly more activity toward C10 acyl-ACP substrates as compared with other medium chain acyl-ACP substrates.

10 Although the resulting *Cuphea* preparation also demonstrates activity against longer-chain substrate in addition to medium-chain fatty acyl-ACP substrate, both above described Bay and *Cuphea* preparations are considered to be "substantially free from other plant proteins" in 15 that they demonstrate a recognizably distinct preferential activity toward medium-chain fatty acyl-ACP substrates. The resulting partially purified preparation(s) may be characterized by various parameters, including but not limited to comparative inhibitor studies and substrate 20 specificity.

As for both the *Cuphea* and Bay preparations, additional and/or alternative purification steps may be desired to purify the protein extract to homogeneity, to increase yield or the like. Moreover, now that the 25 existence of these proteins is confirmed and various properties described, alternative purification protocols and/or additional purification steps are within the capabilities of one skilled in the art.

Other Plant Thioesterases

Also by this invention, sequence information regarding a long-chain thioesterase obtained from safflower (*Carthamus tinctorius*) is described. Interestingly, it has 5 been discovered that at least two of the peptide fragments sequenced from the safflower thioesterase show high sequence identity with portions of the Bay medium-chain preferring thioesterase.

Although described in more detail in the Examples, the 10 safflower thioesterase peptide fragments were obtained upon subjecting acetone ground safflower embryos to an acid precipitation followed by chromatography on an ACP column and a chromatofocusing column. Through analysis of enzyme activity peaks as compared with the proteins obtained from 15 the ACP column, two proteins, one at approximately 34 kD and one at approximately 40 kD were selected for further analysis. Fragments sequenced after cyanogen bromide blotting are shown in Table 9 of Example 14.

In particular, it was found that every fragment 20 sequenced corresponding to the 34 kD band was detected in the 40 kD band. In addition, it appears that the 34 kD product shares the same N-terminus as the 40 kD product. A schematic representation postulating the positioning of various fragments from Table 9 is found in Fig. 5. In 25 addition, it was found that segments of the Bay thioesterase amino acid sequence demonstrated high sequence identity with at least two of the sequenced fragments, S828 and S829.

Genetic Engineering Applications

As is well known in the art, once a plant thioesterase is obtained, it may be used to obtain its corresponding amino acid and/or nucleic acid sequences thereto. As a representative example, the amino acid sequence may be obtained by the sequencing of peptide fragments resulting from partial protease digestion of protein blots recovered from a gel. For sequencing, the use of a two-dimensional gel may be desired over a one dimensional SDS-PAGE gel.

The peptide fragments may be used to deduce amino acid sequences and eventually, amino acid sequence may be obtained. From the amino acid sequence, the information can be reverse translated and, nucleic acid probes can be synthesized for use in PCR process or for use as probes in the recovery of the gene. As yet a different example, the purified protein may be used to raise antibodies thereto. The antibodies, polyclonal or monoclonal, may also be used to isolate other immunologically related plant thioesterase genes. Alternative methods will also be apparent in accordance with methods familiar to those skilled in the art.

The nucleic acid sequences which encode plant thioesterases may be used in various constructs, for example, as probes to obtain further sequences.

Alternatively, these sequences may be used in conjunction with appropriate regulatory sequences to increase levels of the respective thioesterase of interest in a host cell for recovery or study of the enzyme in vitro or in vivo or to decrease levels of the respective thioesterase of interest

for some applications when the host cell is a plant entity, including plant cells, plant parts (including but not limited to seeds, cuttings or tissues) and plants.

A nucleic acid sequence encoding a plant thioesterase 5 of this invention may include genomic, cDNA or mRNA sequence. By "encoding" is meant that the sequence corresponds to a particular amino acid sequence either in a sense or anti-sense orientation. By "extrachromosomal" is meant that the sequence is outside of the plant genome of 10 which it is naturally associated. By "recombinant" is meant that the sequence contains a genetically engineered modification through manipulation via mutagenesis, restriction enzymes, and the like. A cDNA sequence may or may not contain pre-processing sequences, such as transit 15 peptide sequences. Transit peptide sequences facilitate the delivery of the protein to a given organelle and are cleaved from the amino acid moiety upon entry into the organelle, releasing the "mature" sequence. The use of the precursor DNA sequence is preferred in plant cell 20 expression cassettes. Other plastid transit peptide sequences, such as a transit peptide of seed ACP, may be employed to translocate the plant thioesterase of this invention to various organelles of interest. See, U.S. Serial No. 07/437,764, filed 11/15/89 and European Patent 25 Application Publication No. 189,707. In a like manner, once a given plant thioesterase transit peptide is obtained, it may be used to translocate sequences other than its native coding region.

Furthermore, as discussed above the complete genomic sequence of the plant thioesterase may be obtained by the screening of a genomic library with a probe, such as a cDNA probe, and isolating those sequences which regulate expression in seed tissue. In this manner, the transcription and translation initiation regions, introns, and/or transcript termination regions of the plant thioesterase may be obtained for use in a variety of DNA constructs, with or without the thioesterase structural gene. Thus, nucleic acid sequences corresponding to the plant thioesterase of this invention may also provide signal sequences useful to direct transport into a plastid, 5' upstream non-coding regulatory regions (promoters) having useful tissue and timing profiles, 3' downstream 15 non-coding regulatory region useful as transcriptional and translational regulatory regions and may lend insight into other features of the gene.

Once the desired plant thioesterase nucleic acid sequence is obtained, it may be manipulated in a variety of ways. Where the sequence involves non-coding flanking regions, the flanking regions may be subjected to resection, mutagenesis, etc. Thus, transitions, transversions, deletions, and insertions may be performed on the naturally occurring sequence. In addition, all or 25 part of the sequence may be synthesized. In the structural gene, one or more codons may be modified to provide for a modified amino acid sequence, or one or more codon mutations may be introduced to provide for a convenient restriction site or other purpose involved with

construction or expression. The structural gene may be further modified by employing synthetic adapters, linkers to introduce one or more convenient restriction sites, or the like.

5       The nucleic acid or amino acid sequences encoding a plant thioesterase of this invention may be combined with other non-native, or "heterologous", sequences in a variety of ways. By "heterologous" sequences is meant any sequence which is not naturally found joined to the plant  
10      thioesterase, including, for example, combinations of nucleic acid sequences from the same plant which are not naturally found joined together.

The DNA sequence encoding a plant thioesterase of this invention may be employed in conjunction with all or part  
15      of the gene sequences normally associated with the thioesterase. In its component parts, a DNA sequence encoding thioesterase is combined in a DNA construct having, in the 5' to 3' direction of transcription, a transcription initiation control region capable of  
20      promoting transcription and translation in a host cell, the DNA sequence encoding plant thioesterase and a transcription and translation termination region.

Potential host cells include both prokaryotic and eukaryotic cells. A host cell may be unicellular or found  
25      in a multicellular differentiated or undifferentiated organism depending upon the intended use. Cells of this invention may be distinguished by having a plant thioesterase foreign to the wild-type cell present therein,

for example, by having a recombinant nucleic acid construct encoding a plant thioesterase therein.

Depending upon the host, the regulatory regions will vary, including regions from viral, plasmid or chromosomal genes, or the like. For expression in prokaryotic or eukaryotic microorganisms, particularly unicellular hosts, a wide variety of constitutive or regulatable promoters may be employed. Expression in a microorganism can provide a ready source of the plant enzyme. Among transcriptional initiation regions which have been described are regions from bacterial and yeast hosts, such as *E. coli*, *B. subtilis*, *Sacchromyces cerevisiae*, including genes such as beta-galactosidase, T7 polymerase, tryptophan E and the like.

For the most part, the constructs will involve regulatory regions functional in plants which provide for modified production of plant thioesterase, and possibly, modification of the fatty acid composition. The open reading frame, coding for the plant thioesterase or functional fragment thereof will be joined at its 5' end to a transcription initiation regulatory region such as the wild-type sequence naturally found 5' upstream to the thioesterase structural gene. Numerous other transcription initiation regions are available which provide for a wide variety of constitutive or regulatable, e.g., inducible, transcription of the structural gene functions. Among transcriptional initiation regions used for plants are such regions associated with the structural genes such as for nopaline and mannopine synthases, or with napin, ACP

promoters and the like. The transcription/translation initiation regions corresponding to such structural genes are found immediately 5' upstream to the respective start codons. In embodiments wherein the expression of the 5 thioesterase protein is desired in a plant host, the use of all or part of the complete plant thioesterase gene is desired; namely all or part of the 5' upstream non-coding regions (promoter) together with the structural gene sequence and 3' downstream non-coding regions may be 10 employed. If a different promoter is desired, such as a promoter native to the plant host of interest or a modified promoter, i.e., having transcription initiation regions derived from one gene source and translation initiation regions derived from a different gene source, including the 15 sequence encoding the plant thioesterase of interest, or enhanced promoters, such as double 35S CaMV promoters, the sequences may be joined together using standard techniques.

For such applications when 5' upstream non-coding regions are obtained from other genes regulated during seed 20 maturation, those preferentially expressed in plant embryo tissue, such as ACP and napin-derived transcription initiation control regions, are desired. Such "seed-specific promoters" may be obtained and used in accordance with the teachings of U.S. Serial No. 07/147,781, filed 25 1/25/88 (now U.S. Serial No. 07/550,804, filed 7/9/90), and U.S. Serial No. 07/494,722 filed on or about March 16, 1990 having a title "Novel Sequences Preferentially Expressed In Early Seed Development and Methods Related Thereto," which references are hereby incorporated by reference.

Transcription initiation regions which are preferentially expressed in seed tissue, i.e., which are undetectable in other plant parts, are considered desirable for fatty acid modifications in order to minimize any disruptive or 5 adverse effects of the gene product.

Regulatory transcript termination regions may be provided in DNA constructs of this invention as well. Transcript termination regions may be provided by the DNA sequence encoding the plant thioesterase or a convenient 10 transcription termination region derived from a different gene source, for example, the transcript termination region which is naturally associated with the transcript initiation region. Where the transcript termination region is from a different gene source, it will contain at least 15 about 0.5kb, preferably about 1-3kb of sequence 3' to the structural gene from which the termination region is derived.

Plant expression or transcription constructs having a plant thioesterase as the DNA sequence of interest for 20 increased or decreased expression thereof may be employed with a wide variety of plant life, particularly, plant life involved in the production of vegetable oils for edible and industrial uses. Most especially preferred are temperate oilseed crops. Plants of interest include, but are not 25 limited to, rapeseed (Canola and High Erucic Acid varieties), sunflower, safflower, cotton, Cuphea, soybean, peanut, coconut and oil palms, and corn. Depending on the method for introducing the recombinant constructs into the host cell, other DNA sequences may be required.

Importantly, this invention is applicable to dicotyledyons and monocotyledons species alike and will be readily applicable to new and/or improved transformation and regulation techniques.

5       The method of transformation is not critical to the instant invention; various methods of plant transformation are currently available. As newer methods are available to transform crops, they may be directly applied hereunder. For example, many plant species naturally susceptible to  
10      Agrobacterium infection may be successfully transformed via tripartite or binary vector methods of *Agrobacterium* mediated transformation. In addition, techniques of microinjection, DNA particle bombardment, electroporation have been developed which allow for the transformation of  
15      various monocot and dicot plant species.

In developing the DNA construct, the various components of the construct or fragments thereof will normally be inserted into a convenient cloning vector which is capable of replication in a bacterial host, e.g., *E. coli*. Numerous vectors exist that have been described in the literature. After each cloning, the plasmid may be isolated and subjected to further manipulation, such as restriction, insertion of new fragments, ligation, deletion, insertion, resection, etc., so as to tailor the  
20      components of the desired sequence. Once the construct has been completed, it may then be transferred to an appropriate vector for further manipulation in accordance  
25      with the manner of transformation of the host cell.

Normally, included with the DNA construct will be a structural gene having the necessary regulatory regions for expression in a host and providing for selection of transformant cells. The gene may provide for resistance to 5 a cytotoxic agent, e.g. antibiotic, heavy metal, toxin, etc., complementation providing prototrophy to an auxotrophic host, viral immunity or the like. Depending upon the number of different host species the expression construct or components thereof are introduced, one or more 10 markers may be employed, where different conditions for selection are used for the different hosts.

It is noted that the degeneracy of the DNA code provides that some codon substitutions are permissible of DNA sequences without any corresponding modification of the 15 amino acid sequence. When any non-plant derived DNA sequence is to be expressed in a plant host cell, the use of "plant preferred codons" is desirable.

As mentioned above, the manner in which the DNA construct is introduced into the plant host is not critical 20 to this invention. Any method which provides for efficient transformation may be employed. Various methods for plant cell transformation include the use of Ti- or Ri-plasmids, microinjection, electroporation, DNA particle bombardment, liposome fusion, DNA bombardment or the like. In many 25 instances, it will be desirable to have the construct bordered on one or both sides by T-DNA, particularly having the left and right borders, more particularly the right border. This is particularly useful when the construct uses *A. tumefaciens* or *A. rhizogenes* as a mode for

transformation, although the T-DNA borders may find use with other modes of transformation.

Where *Agrobacterium* is used for plant cell transformation, a vector may be used which may be 5 introduced into the *Agrobacterium* host for homologous recombination with T-DNA or the Ti- or Ri-plasmid present in the *Agrobacterium* host. The Ti- or Ri-plasmid containing the T-DNA for recombination may be armed (capable of causing gall formation) or disarmed (incapable 10 of causing gall formation), the latter being permissible, so long as the vir genes are present in the transformed *Agrobacterium* host. The armed plasmid can give a mixture of normal plant cells and gall.

In some instances where *Agrobacterium* is used as the 15 vehicle for transforming plant cells, the expression construct bordered by the T-DNA border(s) will be inserted into a broad host spectrum vector, there being broad host spectrum vectors described in the literature. Commonly used is pRK2 or derivatives thereof. See, for example, 20 Ditta et al., *PNAS USA*, (1980) 77:7347-7351 and EPA 0 120 515, which are incorporated herein by reference. Included with the expression construct and the T-DNA will be one or more markers, which allow for selection of transformed *Agrobacterium* and transformed plant cells. A number of 25 markers have been developed for use with plant cells, such as resistance to chloramphenicol, the aminoglycoside G418, hygromycin, or the like. The particular marker employed is not essential to this invention, one or another marker

being preferred depending on the particular host and the manner of construction.

For transformation of plant cells using *Agrobacterium*, explants may be combined and incubated with the transformed 5 *Agrobacterium* for sufficient time for transformation, the bacteria killed, and the plant cells cultured in an appropriate selective medium. Once callus forms, shoot formation can be encouraged by employing the appropriate plant hormones in accordance with known methods and the 10 shoots transferred to rooting medium for regeneration of plants. The plants may then be grown to seed and the seed used to establish repetitive generations and for isolation of vegetable oils.

The invention now being generally described, it will 15 be more readily understood by reference to the following examples which are included for purposes of illustration only and are not intended to limit the present invention.

#### EXAMPLES

20 In the experimental disclosure which follows, all temperatures are given in degrees centigrade ( $^{\circ}$ ), weights are given in grams (g), milligram (mg) or micrograms ( $\mu$ g), concentrations are given as molar (M), millimolar (mM) or micromolar ( $\mu$ M) and all volumes are given in liters (l), 25 microliters ( $\mu$ l) or milliliters (ml), unless otherwise indicated.

##### Example 1 - C12-Preferring Acyl-ACP Thioesterase Assay

To assay for C12 thioesterase activity the following mixture is incubated at  $30^{\circ}$  for 30 min: "buffer"

comprising 7mM KH<sub>2</sub>PO<sub>4</sub>-KOH pH 8, 20% v/v glycerol, 1mM dithiothreitol (DTT), 0.1% v/v Triton X100; sample to be tested for activity in the same or similar buffer as the "extraction buffer" described in Example 2; and 5μl of <sup>14</sup>C-5 radiolabeled lauroyl-ACP substrate for a total volume of 100μl and final lauroyl-ACP concentration of 0.5μM.

Lauroyl-ACP substrate is prepared according to the method of Rock et al (*Methods in Enzymology* (1981) 72:397-403), using ACP prepared from *Escherichia coli* by the method of 10 Rock and Cronan (*Methods in Enzymology* (1981) 71:341-351). The laurate is radiolabeled in the carboxylate group at a specific radioactivity of 50-60μCi/μmol.

The reaction is stopped by adding 0.5ml cold (0°) 10% v/v acetic acid. The fatty acid product of the hydrolytic 15 enzyme action is extracted away from the unhydrolyzed substrate by adding 1ml diethyl ether and mixing vigorously. After settling for a few minutes the upper ether layer is transferred to 5ml scintillation fluid for determination of radioactivity by liquid scintillation spectrometry. Additional ether extractions may be performed to recover remaining traces of the reaction product for more accurate quantitation of the enzyme activity if desired. The amount of ether-extracted radioactivity is a direct measure of C12-preferring acyl-20 ACP thioesterase activity, provided the amount of enzyme is not sufficient to hydrolyze more than about 25% of the substrate. With greater activity than this the relationship between radioactivity in the ether layer and the quantity of enzyme becomes markedly nonlinear. The

enzyme preparation must then be diluted appropriately to bring the activity into the linear range of the assay.

The activity is confirmed to be thioesterase by analysis of the ether-soluble product using thin-layer chromatography (TLC). The product co-migrates with authentic laurate on a silica TLC plate (solvent: 80% hexane, 20% diethyl ether, 1% acetic acid v/v). If phenacyl esters are prepared (Borch, R.F., *Analytical Chemistry* (1975) 47:2437-2439) using the ether product-  
containing layer from the assay procedure, the resulting radioactive spot co-migrates with authentic lauroyl phenacyl ester on a C18 TLC plate (solvent: 100% methanol), as does the product of base hydrolysis of the lauroyl-ACP substrate. These observations verify that the ether-extracted product of the enzyme reaction is free laurate.  
It is also deduced that the enzyme of interest hydrolyzes the thioester bond, e.g. it cannot be a protease attacking the ACP moiety of the substrate or the product would be lauroyl-phosphopantetheine which would have migrated differently on TLC.

Example 2 - Bay Thioesterase Purification & Identification

Immature seeds of *Umbellularia californica* ("Bay") are harvested at the stage at which decanoate and laurate predominate in the fatty acid composition as determined by total fatty acid analysis of the cotyledons. The cotyledons from such seeds are dissected from the other seed parts and stored frozen at -70°. This comprises the source tissue for enzyme extraction.

The frozen cotyledons are powdered in a stainless steel mortar and pestle at approximately -70°, and the powder is stored under liquid nitrogen or at -70° until required. Extraction is accomplished by adding, at 0-4°,

5 to the powder 4ml/g of "extraction buffer" comprising 50mM KH<sub>2</sub>PO<sub>4</sub>-KOH pH 6.9, 5mM ethylenediamine tetraacetate (EDTA), 2mM DTT, 1mM sodium ascorbate, 1mM phenylmethysulfonyl fluoride, 1μM leupeptin, and 1μM pepstatin. The stirred mixture of powder and buffer is blended in a motorized

10 macerator (Brinkmann (Westbury, NY) "Polytron", three bursts of 45 sec each) and then filtered through four layers of cheesecloth. This and all subsequent steps are conducted at 0-4°. The resulting filtrate is centrifuged at approximately 14,000xg (max.) for 30 min. The

15 supernatant fractions are filtered through "Miracloth" (Calbiochem. Corp., LaJolla, CA) and the resulting liquid is referred to as the "crude extract".

The crude extract is subjected to ammonium sulfate fractionation as follows. Sufficient solid ammonium sulfate

20 is gradually added with stirring over 30 min to achieve 70% saturation. The preparation is then stirred for a further 30 min. After centrifuging as described above, the pelleted material is resuspended in extraction buffer (2ml/g original tissue weight) and stirred for 10 min until

25 dissolved. Ammonium sulfate is then added as before, but this time only sufficient to achieve 50% saturation. After centrifuging as before, the supernatant fraction is discarded. The pelleted material, which contains the C12-preferring acyl-ACP thioesterase, may be frozen by

immersion in liquid nitrogen and then stored at -70° at this stage if desired. The resulting material is referred to as the "ammonium sulfate fraction." Very little of the C12-preferring acyl-ACP thioesterase activity is lost if 5 the pellet is frozen very rapidly.

After thawing to 4° if necessary, the pellet material is resuspended in "HAI buffer" (1ml/g original tissue weight), comprising 50mM KH<sub>2</sub>PO<sub>4</sub>-KOH pH 6.9, 25% w/v glycerol, 1mM DTT. The resuspended preparation is placed 10 in dialysis tubing (12,000-14,000 molecular weight cutoff) and set to dialyze against HAI buffer. (Typically a preparation from 600g of cotyledon tissue will require two successive dialysis steps against 4 liters of buffer each, for at least three hours each.) Before application to the 15 first column, the dialyzed material is centrifuged as described above and the pelleted material is discarded.

The supernatant material from post-dialysis centrifugation is applied to a column of hydroxyapatite (HA-Ultrogel from IBF Biotechnics, catalog. no. 247741, 20 Savage, MD; for a preparation from 500-1200g of tissue typically 10cm diameter x 12.5cm bed height), equilibrated in HAI buffer. The column is then washed with HAI buffer until the absorbance of the effluent at 280nm no longer changes. A considerable amount of protein and sometimes a 25 small amount of the C12-preferring acyl-ACP thioesterase activity fail to bind the column and are washed through it. The bulk of the C12-preferring acyl-ACP thioesterase activity binds, and is eluted by applying "HA2 buffer" comprising 400mM KH<sub>2</sub>PO<sub>4</sub>-KOH pH 6.9, 25% w/v glycerol, 1mM

DTT. The effluent is collected in fractions (5-10 ml in volume), which are then assayed for C12-preferring acyl-ACP thioesterase activity. The active fractions are combined and dialyzed as described above, against "CM1 buffer" comprising 5mM KH<sub>2</sub>PO<sub>4</sub>-KOH pH 6.5, 25% w/v glycerol, 1mM EDTA, 1mM DTT (typically three dialysis periods of at least 3 hr each against 4 liters each). After dialysis the material is clarified by centrifugation as described previously, the pellets being discarded.

10       The supernatant fraction is then applied to a cation exchange column (Pharmacia CM-Sepharose Fast Flow, Piscataway, NJ, catalog no. 17-0719-01, 10cm diameter x 14cm bed height) equilibrated with CM1 buffer. After loading, the column is washed with CM1 buffer until the 15 absorbance of the effluent stream at 280nm no longer changes. A considerable quantity of protein and a significant amount (e.g. 50%) of the C12-preferring acyl-ACP thioesterase activity fail to bind the column and are washed through it. This partial binding of the C12-preferring acyl-ACP thioesterase has been investigated and found to result from aggregation of this enzyme with other, unidentified proteins at the time of extraction. In effect there are two populations of the C12-preferring acyl-ACP thioesterase up to this point in the purification scheme, 20 free enzyme and aggregate. The cation exchange column separates these two forms and the aggregate is discarded. The unaggregated form of the C12 acyl-ACP thioesterase is eluted from the column by applying "CM2 buffer" comprising 25 50mM KH<sub>2</sub>PO<sub>4</sub>-KOH pH 6.9, 150mM NaCl, 25% w/v glycerol, 1mM

EDTA, 1mM DTT. The effluent stream is fractionated and assayed as before, and the active fractions are pooled and dialyzed against "ACP1 buffer" comprising 10mM KH<sub>2</sub>PO<sub>4</sub>-KOH pH 6.5, 150mM NaCl, 25% w/v glycerol, 1mM EDTA, 1mM DTT, 5 0.1% w/v 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS). Typically two successive dialyses for at least 3 hr each, against 4 liters each, suffice for a preparation from 600g tissue.

The dialyzed material is then applied to a column of 10 immobilized ACP (2.5cm diameter x 10.5cm bed height). This column is manufactured by coupling *Escherichia coli* ACP to cyanogen bromide-activated Sepharose 4B according to instructions supplied by the manufacturer of this column packing (Pharmacia Inc., Piscataway, NJ). The *E. coli* ACP 15 is prepared as referenced in Example 1. Before use the column is equilibrated with ACP1 buffer. The dialyzed material from the cation exchange column is applied at 1-1.3ml/min, and fractions of 8ml volume are collected throughout the procedure. Fractions are assayed for C12- 20 preferring acyl-ACP thioesterase activity, and for total protein content using a Coomassie Blue assay method (Bio-Rad Inc., Richmond, CA, catalog no. 500-0001). A substantial amount of protein washes through the column without binding. Almost all of the C12-preferring acyl-ACP 25 thioesterase activity binds. The column is washed with ACP1 buffer until the protein assay detects no more protein in the effluent stream. It is then washed with "ACP2 buffer" comprising 50mM KH<sub>2</sub>PO<sub>4</sub>-KOH pH 8.5, 50mM glycine, 25% w/v glycerol, 1mM EDTA, 0.1% w/v CHAPS. This

high pH wash serves to remove nonspecifically bound protein. A small amount of C12 acyl-ACP thioesterase activity is occasionally co-eluted with it. After the protein assay has again indicated that no more protein is being eluted, a linear "elution gradient" is applied. This comprises 560ml combined volume of "ACP3 buffer" (100mM KH<sub>2</sub>PO<sub>4</sub>-KOH pH 6.9, 25% w/v glycerol, 1mM EDTA, 1mM DTT, 0.1% w/v CHAPS) and "ACP4 buffer" (500mM KH<sub>2</sub>PO<sub>4</sub>-KOH pH 6.9, 25% w/v glycerol, 1mM EDTA, 1mM DTT, 0.1% w/v CHAPS). If C12-preferring acyl-ACP thioesterase activity is still eluting from the column when the gradient ends, its elution can be completed by applying more ACP4 buffer. The collected fractions are assayed as before, and a second C12-preferring acyl-ACP thioesterase assay is also performed with the fractions diluted fifty-fold. By compensating for nonlinearity of the assay this gives a more precise location of the maximum enzyme activity. The C12-preferring acyl-ACP thioesterase activity is typically present in the gradient-eluted fractions as two peaks, a smaller one eluting just before a much larger one.

The fractions comprising each peak are pooled separately. The larger, later eluting peak is the most pure material that is used for subsequent experiments, protein sequencing etc. Analysis of this material by typical SDS-PAGE procedures shows only 5-6 strongly staining bands including a band of an approximate molecular weight at 34 kD and a few weakly staining ones.

Aliquots of fractions from the ACP column are analyzed by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and

silver staining. The band pattern at the peak of eluted activity is markedly simplified relative to the flow-through and pH 8.5-eluted material. Band patterns are compared from fraction to fraction to identify bands whose 5 intensities increase and decrease in concert with 12:0-ACP thioesterase activity. One band pattern corresponding to an approximate molecular weight of 34 kD, satisfied this criterion. In some preparations a closely spaced doublet is seen at this position on the SDS gel.

10 Alternatively, a variety of chromatographic and electrophoretic techniques may be applied to the substantially purified 12:0-ACP thioesterase pool from the ACP column, including ion-exchange chromatography, immobilized dye chromatography, and native gel 15 electrophoresis. None of them purifies the enzyme to electrophoretic homogeneity. However, in all cases a band or pair of bands of approximate molecular weight 34 kD co-elutes or co-migrates with the enzyme activity. The best resolution is obtained by chromatography on S-Sepharose 20 followed by Blue 4 agarose, with the most informative separation occurring on the final Blue 4 agarose column. The most abundant eluted proteins are those of approximate molecular weight 65 kD, 39 kD, and 34 kD (doublet). Only the 34 kD pair elutes in synchrony with the peak of 12:0- 25 ACP thioesterase activity.

Example 3 - C12-preferring acyl-ACP Thioesterase Inhibitor Studies

Table 1 below reports inhibition of Bay cotyledon C12- 30 preferring acyl-ACP thioesterase by thiol reagents observed

when an ammonium sulfate fraction (see, Example 2) was assayed (see, Example 1).

Table 1

	<u>Addition to Assay</u>	<u>Mean Activity*</u>	<u>Percent Inhibition</u>
5	None (control)	4322	-
	0.5mM iodoacetamide	4180	3
10	5mM	4047	6
	0.5mM N-ethylmaleimide	4320	0
	5mM	103	98

\*"Mean Activity" is a measurement of the mean score of duplicates provided in cpm as observed in the ether layer of Example 1

After removal of dithiothreitol from an ammonium sulfate fraction preparation by passage through a small column of G25-50 gel filtration medium (Pharmacia, Piscataway, NJ) the following assay results were observed.

Table 2

	<u>Addition to Assay</u>	<u>Mean Activity*</u>	<u>Percent Inhibition</u>
25	None (control)	3776	-
	5mM iodoacetamide	3851	0
	5mM N-ethylmaleimide	269	93

30 \*"Mean Activity" is a measurement of the mean score of duplicates provided in cpm as observed in the ether layer of Example 1

These preliminary inhibitor studies indicate that the Bay C12-preferring acyl-ACP thioesterase is insensitive to

5mM iodoacetamide and almost completely inhibited by 5mM N-ethylmaleimide. These results suggest that C12-preferring acyl-ACP thioesterase is an "active thiol" type of esterase rather than an "active serine" type.

5

Example 4 - Bay C12-preferring Acyl-ACP Thioesterase Substrate Specificity as a Function of Chain Length

In tests comparing activity of the ammonium sulfate fraction preparations of Bay C12-preferring acyl-ACP thioesterase of Example 2 against various length medium-chain fatty acids in the assay of Example 1, the greatest activity has been manifest towards C12-ACP over C8, C10, C12, C14 and C16 ACP substrates as shown in Table 3.

15

Table 3

	<u>Acyl-ACP acyl Chain length</u>	<u>Relative Thioesterase Activity*</u>
20	8	1.0
	10	2.7
	11	3.7
	12	24.0
25	14	4.0
	16	4.7

\*C8-ACP activity set to 1.0

30 Example 5 - Bay C12-preferring Thioesterase Substrate Specificity as a Function of ACP versus CoA

Crude extracts of Bay cotyledons hydrolyze lauroyl coenzyme A (CoA) as well as lauroyl-ACP. This is due to

the presence of separate enzymes acting respectively on these substrates, i.e. to C12-preferring acyl-ACP thioesterase acting on lauroyl-ACP and another enzyme acting on lauroyl CoA. The distinct nature of these 5 enzymes is indicated by their separation at the ACP column stage in the purification scheme. Lauroyl-CoA hydrolysis activity is found chiefly in the material which fails to bind the ACP column, and C12-preferring acyl-ACP thioesterase activity is found in the material which binds 10 and which is subsequently eluted with a phosphate concentration gradient. Activities of the peak fraction of unbound and bound material serves to illustrate this separation, as shown in the following table.

Table 4

15	Fraction	Activity on C12-CoA Substrate*	Activity on C12-ACP Substrate*
	Flow-through (nonbinding)	10808	300
20	Gradient-eluted	27	2772

\*cpm of ether-extractable product

Therefore, the Bay C12-preferring acyl-ACP thioesterase shows much more activity towards lauroyl-ACP than towards lauroyl-CoA.

25

#### Example 6 - Role of the Enzyme in Laurate Production

Further evidence that the C12-preferring acyl-ACP thioesterase is involved in the biosynthesis of laurate groups that predominate in the Bay seeds comes from a 30 comparison of the extractable activity of the enzyme at two different stages of seed development. As shown in the

following table, Table 5, very young seeds, which contain only long-chain fatty acids and insignificant amounts of laurate, yield much less C12-preferring ACP thioesterase than older seeds that have accumulated significant amounts of laurate. Thus it appears that significant activity of this enzyme is only present when the seeds are accumulating laurate. Additionally, there appears to be much less difference in lauroyl-CoA hydrolysis activity, consistent with their being different enzymes as discussed above in

10 Example 5.

Table 5

<u>Tissue Source</u>	<u>C12 acyl-CoA Hydrolysis Activity In Assays*</u>	<u>C12 acyl-ACP Thioesterase Activity In Assays*</u>
15 Normal Seeds ( $\approx$ 2g/cotyledon pair)	31,268	4704
20 Young Seeds ( $\approx$ 0.5g/cotyledon pair)	29,995	376

\*cpm ether-extractable radioactivity

25 Example 7 - In vitro Bay Fatty Acid Synthesis Assay

An ammonium sulfate fraction of a Bay embryo extract will synthesize the same specific fatty acids as those found in the maturing seed if supplied with *E. coli* ACP, malonyl-CoA, and other typical cofactor and substrate requirements of documented *in vitro* fatty acid synthesizing systems (Jaworski, et al., *Arch. Biochem. Biophys.* (1974) 163:769-776). The products of this *in vitro* activity include water-soluble octanoyl and decanoyl esters but almost undetectable water-soluble lauroyl ester, even

though laurate is the major free fatty acid product. These results are most simply explained in terms of the fatty acid synthesizing system producing acyl-ACP's of successively increased chain length, and the specific 5 lauroyl-ACP thioesterase intercepting the acyl-ACP when the acyl moiety has been extended as far as twelve carbon atoms, by hydrolyzing apart the acyl and ACP moieties at that stage.

10 Example 8 - C-10 Preferring Acyl-ACP Thioesterase Assay

Following the same general procedures as outlined in Example 1, to assay for C10 thioesterase activity, the following mixture is incubated at 30° for 10-60 min: 50 $\mu$ l sample to be tested in the same or similar "extraction 15 buffer" described in Example 9A, and approximately 250pmol of [ $^{14}$ C]-radiolabeled acyl-ACP substrate, (usually decanoyle-ACP is labeled in the carboxylate group to 50- 60 $\mu$ Ci/ $\mu$ mol) in a total volume of 50 $\mu$ l, for a final decanoyle-ACP concentration of 0.5-5.0 $\mu$ M, typically 5.0 $\mu$ M. 20 The reaction is stopped by adding 0.5ml 10% (v/v) cold (4°) acetic acid and placing the reaction mixture on ice for a few minutes. The fatty acid product of the hydrolytic enzyme action is extracted away from the unhydrolyzed substrate by adding 2ml diethyl ether and mixing 25 vigorously. The ether is transferred to 5ml scintillation fluid for scintillation counting. Additional ether extracts may be performed to recover remaining traces of product for more accurate quantitation of the activity if desired.

Example 8A

In alternative to Example 8, enzyme activity is assayed by adding 25 $\mu$ l of sample to a screw-cap glass vial. Next, concentrated radiolabelled substrate [ $^{14}\text{C}$ ]-C10:0-ACP, 5 54.7 $\mu\text{Ci}/\mu\text{mol}$  is added to the vial so that the substrate concentration will be 0.5 $\mu\text{M}$  in the final 100 $\mu\text{l}$  assay volume. Finally enough assay buffer (100mM glycine-HCl, pH 9, 0.2% CHAPS, 10mM  $\beta$ -mercaptoethanol) is added to the vial so that the total volume is 100 $\mu\text{l}$ . The mixture is allowed 10 to react by incubating at 30°C for 30 minutes. The reaction is stopped by adding 0.5ml of 10% (v/v) acetic acid and then 1ml diethyl ether (anhydrous). The radiolabelled free fatty acid product is extracted by vigorously mixing the stopped reaction. The ether phase is 15 then transferred to 5ml of scintillation fluid and radioactivity determined by liquid scintillation counting.

Example 9 - Cuphea C10 Preferring Acyl-ACP Thioesterase Purification and Identification

20 Immature seeds of *Cuphea hookeriana* are harvested. The total fatty acid composition of a few of the harvested seeds is analyzed by standard techniques to make sure that they are at the correct stage of development. This is defined as the stage at which octanoate and decanoate 25 predominate in the fatty acyl composition. The harvested seeds are stored frozen at -70°. This comprises the source tissue for enzyme extraction.

Example 9A

A first method for the purification and identification of a *Cuphea* C10-preferring acyl-ACP thioesterase is provided.

5 An acetone powder is prepared by grinding the seeds to a powder in a mortar and pestle under liquid nitrogen, and then grinding the powder in a mortar and pestle with cold acetone (at approximately -20°). The powder is collected by filtration and rinsed with cold ether to remove  
10 remaining traces of acetone. It is then extracted with 10ml of "extraction buffer" per gram of acetone powder weight (this and all subsequent steps at 0-4°) comprising 50mM KH<sub>2</sub>PO<sub>4</sub>-KOH pH 7.5, 10mM 2-mercaptoethanol. The homogenate is centrifuged at 11,000xg for 15 min at 4°, and  
15 the supernatant fraction used for subsequent purification steps after filtration through two layers of Miracloth (Calbiochem. Inc.; LaJolla, CA).

The supernatant fraction is then subjected to ammonium sulfate fractionation. The 40-60% saturation ammonium sulfate pellet (prepared as described in Example 2) is redissolved in "buffer" comprising 50mM KH<sub>2</sub>PO<sub>4</sub>-KOH pH 6.9, 10% (v/v) glycerol, and 10mM 2-mercaptoethanol, and dialyzed against this buffer to remove remaining ammonium sulfate.

25 The resulting preparation is then subjected to hydroxyapatite column chromatography. The following method applies to ammonium sulfate fraction from 100g fresh weight of starting seed tissue. The dialyzed ammonium sulfate fraction (35-40ml) is applied to a column of hydroxyapatite

(2.5cm x 14cm bed height of Bio-Gel HTP from Bio-Rad Inc.; Richmond, CA, catalog no. 130-0420), equilibrated in 50mM KH<sub>2</sub>PO<sub>4</sub>-KOH pH 6.9, 10% (v/v) glycerol, 4mM 2-mercaptoethanol. The column is then washed (flow rate 5 1.5ml/min throughout) with 280ml of the same buffer. Elution is accomplished with a 580ml linear gradient from these conditions to 350mM KH<sub>2</sub>PO<sub>4</sub>-KOH pH 6.9, 10% (v/v) glycerol, 4mM 2-mercaptoethanol, collecting fractions of 12ml volume. The eluted fractions are assayed for 10 hydrolase activity using decanoyl-ACP as substrate.

Two peaks of activity are obtained, one passing through the column without binding, and the other binding and being subsequently eluted with the phosphate gradient. Both peaks from the hydroxyapatite column contain 15 hydrolytic activity towards long-chain substrates (acyl group of 14 or more carbon atoms). As far as the medium-chain substrates are concerned, the flow-through peak shows little preference, whereas the gradient peak shows considerable preference for decanoyl-ACP (See, Example 20 11A).

At an early stage in the partial purification, when buffered with 100mM HEPES, the decanoyl-ACP C10-preferring acyl-ACP thioesterase shows considerable tolerance of assay pH, activity changing minimally between pH 6.5 and 8.5, 25 with a maximum at pH 7.5. In contrast there is sensitivity to ionic strength in the assay, e.g. using potassium phosphate as the assay buffer activity declines as the phosphate concentration is raised, although activity is still detectable in 350 mM phosphate.

The C10-preferring acyl-ACP thioesterase activity and other proteins in the partially purified preparations are lowered in concentration by extensive contact with glass and plastic surfaces. This effect is reduced by the 5 inclusion of detergents such as Triton X100 or CHAPS in the column and assay buffers. Some detergents are stimulatory in the assay.

The C10-preferring acyl-ACP thioesterase activity is rapidly lost during the ammonium sulfate precipitation step 10 of purification unless 2-mercaptoethanol is present in the buffers as described above. In the buffers described the activity is very stable both at 0° and during repeated freezing to -20° or -70°.

Example 9B

15 As a more preferred alternative to Example 9A, seeds are extracted as follows.

An extraction paste is made with 1375ml of extraction buffer (200mM Bis-Tris-HCl, pH 6.5, 10mM β-mercaptoethanol), 100g polyvinylpolypyrrolidone, and 13.75g 20 soluble polyvinylpyrrolidone (10,000 average molecular weight). 100g of *Cuphea* seeds are added to the paste. All subsequent steps are performed at 4°C. The seeds and paste are homogenized with a Polytron until the mixture is smooth and there are no whole seeds intact. The homogenate is 25 centrifuged at 10,000xg for 20 minutes. The supernatant is decanted and filtered through Miracloth.

The filtered supernatant is mixed into a slurry with 100ml of the settled Blue-4 agarose resin that has been equilibrated with the extraction buffer. The slurry is

washed on a Buchner funnel with 500ml of extraction buffer, then poured into a glass column and rinsed with more extraction buffer until the resin is packed. The column is first washed with 100mM NaCl, 200mM Bis-Tris-HCl, pH 6.5,

5 10mM  $\beta$ -mercaptoethanol. 400mM NaCl, 200mM Bis-Tris-HCl, pH 6.5, 10mM  $\beta$ -mercaptoethanol is applied to the column and the eluate collected in fractions. Those fractions having enzyme activity are pooled and dialyzed against "S buffer" (50mM Bis-Tris-HCl, pH 6.0, 0.2% (w/v) CHAPS, 10mM  $\beta$ -

10 mercaptoethanol).

Next the sample is chromatographed on an S-Sepharose column as follows. The dialyzed sample from the Blue-4 column is loaded on a 50ml column of S-Sepharose resin that has been equilibrated with S buffer. After washing the 15 column with more S-buffer, the column is rinsed with 200mM NaCl, 50mM Bis-Tris-HCl, pH 6.0, 0.2% (w/v) CHAPS, 10mM  $\beta$ -mercaptoethanol. Those fractions having enzyme activity are pooled and dialyzed a second time against S buffer.

Next the sample is chromatographed on a Pharmacia FPLC 20 (Piscataway, NJ) Mono-S column as follows. The dialyzed sample from the S-sepharose column is loaded on a 1ml Mono-S column that has been equilibrated with S buffer. The column is washed with S-buffer until the 280nm absorbance has leveled. A 45ml gradient is applied to the column 25 using S-buffer and S-buffer containing NaCl. The activity elutes between 75mM and 150mM NaCl. Those fractions with enzyme activity are pooled and dialyzed a third time against S buffer.

Finally the sample is chromatographed on an ACP column as follows. A column containing 15ml of acyl-carrier protein coupled to Sepharose is equilibrated with S-buffer. The dialyzed sample from the Mono-S column is loaded onto 5 the ACP column at 0.2ml/min. The column is washed with S-buffer until the 280nm absorbance has leveled into a baseline. A 130ml gradient is applied to the column using S-buffer and S-buffer containing NaCl. The activity elutes between 50mM and 80mM NaCl. Those fractions having enzyme 10 activity are pooled.

Example 9C

As a more preferred alternative to Example 9A or 9B, forty grams of polyvinylpolypyrrrolidone (PVPP) are mixed with 550 ml "extraction buffer" comprising 200 mM bis-Tris-HCl pH 6., 10mM 2-mercaptoethanol, 1% (w/v) polyvinylpyrrolidone-10. To this mixture are added 40g frozen *Cuphea* seeds. The mixture is then blended in a Polytron homogenizer until no intact seeds remain and the slurry is smooth. This and all subsequent steps are 15 conducted at 0-4°C. The preparation is centrifuged at 12,000xg for 20 min and further clarified by filtration 20 through Miracloth.

This preparation is mixed with 100ml of settled hydroxyapatite which has been equilibrated with "buffer A" 25 (50mM bis-Tris-HCl pH 6., 10μM 2-mercaptoethanol). Three extract volumes of 10mM 2-mercaptoethanol are then added slowly over 30 min, with constant stirring. The hydroxyapatite gel is collected on a sintered glass funnel and rinsed with buffer A until the effluent is colorless.

The collected hydroxyapatite is then transferred to a column and further rinsed with buffer A at 2ml/min until the column is packed. A 400ml elution gradient is applied (2ml/min), from buffer A to buffer B (300mM potassium phosphate pH 6.9 in buffer A). Effluent fractions are assayed for hydrolysis of 10:0-ACP. Two overlapping peaks of activity are obtained. The fractions comprising the later-eluting peak are pooled and dialyzed against buffer A.

10 The dialyzed material is applied at 1.3ml/min to a 2.5 x 6.5cm column of Blue 4 Agarose (Sigma Chemical Co.; St. Louis, MO) equilibrated with buffer A. The column is washed with buffer A, and enzyme activity is subsequently eluted with a 400ml gradient from buffer A to buffer C (buffer A containing 1M NaCl). The eluted fractions contain three peaks of 10:0-ACP hydrolysis activity. Those fractions comprising the second peak to elute (eluted by approximately 0.4m NaCl) are pooled and dialyzed against buffer A.

20 The dialyzed material is applied at 0.5ml/min to a 1.7 x 6cm column of immobilized 10:0-ACP analog equilibrated with buffer A. (This column is prepared by reacting heptylamine with iodoacetic anhydride in diethyl ether, and adding the product to purified, reduced *E. coli* ACP. The residual reagents are removed by gel filtration chromatography and the resulting 10:0-ACP analog is coupled to Pharmacia CNBr-activated Sepharose per the manufacturer's directions, blocking unreacted groups with Tris.) The column is rinsed with buffer A and then

activity is eluted using a 200ml gradient from buffer A to buffer D (buffer A containing 0.5M NaCl). Fractions corresponding to the eluted peak of 10:0-ACP activity are pooled and dialyzed against 50mM bis-Tris pH 6, 10mM 2-  
5 mercaptoethanol, 0.2% (w/v) CHAPS, 5mM sodium ascorbate.

Example 9D

The protocol described in Example 9C may be further modified as follows. The fractions corresponding to the eluted peak of 10:0-ACP activity are pooled and dialyzed  
10 against buffer E (50mM Bis-Tris-HCl pH 6.0, 0.2% CHAPS,  
10mM  $\beta$ -mercaptoethanol). An FPLC Mono-S column (Monos®  
HR5/5, Pharmacia LKB Biotechnology, NJ) is equilibrated  
with the buffer E. The dialyzed pool is loaded onto the  
column. All the C10 and C18:1 activity appears to bind to  
15 the column. The activities may be eluted with a linear  
140ml gradient from (buffer) to (buffer+1M NaCl).

C18:1 activity elutes between 75mM and 100mM NaCl.  
There is a second peak of activity that elutes between  
150mM and 175mM NaCl. The second activity peak is  
20 primarily C10 and C18:1 activity, with relatively little  
C12, C14, or C16 activity. Any C18:1 activity in the  
second peak could be due to contamination by residual C18:1  
activity.

25 Example 10 - C10 Acyl-ACP Thioesterase Inhibitor Studies

Preliminary inhibitor studies with material from Example 9A indicate that the Cuphea C10-preferring acyl-ACP thioesterase is insensitive to phenylmethylsulfonyl fluoride, insensitive to iodoacetamide, and completely

inhibited by 5mM N-ethylmaleimide. This suggests that it is an "active thiol" type of esterase rather than an "active serine" type.

5    Example 11 - Cuphea C10 Acyl ACP Thioesterase Substrate Specificity as a Function of Chain Length

Example 11A

The substrate specificity of Cuphea C10 acyl-ACP thioesterase towards medium-chain acyl-ACP's has been  
10 determined at the hydroxyapatite stage in purification, as described in Example 9A:

Table 6

	<u>Substrate</u>	<u>Hydrolysis Activity (mean) (pmol/min)</u>
15	C6-ACP	188
	C8-ACP	485
	C10-ACP	6950
	C11-ACP	649
	C12-ACP	1032
	C14-ACP	4055

The activity towards the longer-chain substrate 14:0-ACP is considered to be due to the presence of long-chain thioesterase activity, analogous to long-chain thioesterases of safflower seed and avocado mesocarp tissue  
25 that are described in published literature. Assay of the preparation with the preferred substrate of such an enzyme, 18:1-ACP, indicates the presence of substantial activity, consistent with this hypothesis. The activity towards 10:0-ACP and the smaller amount of activity towards 8:0-ACP  
30 indicate the presence of the medium-chain-specific thioesterase responsible for medium-chain fatty acid production in developing *Cuphea hookeriana* seeds.

The reactions catalyzed have been shown to be simple hydrolysis. The ether-extracted products of both "time zero" reactions and one hour reactions with 6:0-ACP, 8:0-ACP, and 10:0-ACP substrates were chromatographed on silica G thin-layer plates (mobile phase: hexane/diethyl ether/acetic acid, 80:20:1 v/v) to determine the lipid class. Lauric acid was added as unlabeled carrier to inhibit evaporation of liberated short-chain free fatty acids. Tricaprin, dicaprin, monocaprin, and lauric acid were used as standards. The TLC plate was developed half-way and then air dried for 5 minutes. The plate was then returned to the tank and development was completed to within 1cm of the top of the plate. The developed plate was dried and then scanned for 800 mins on an AMBIS (AMBIS Systems, Inc. San Diego, CA) radiochromatogram scanner to quantitate radiolabeled spots. Following scanning, the plate was stained in iodine vapor for 15 minutes to visualize the lipids. The principal radiolabeled products co-migrated with the free fatty acids, and were substantially more radioactive in the samples incubated for 1 hour compared with the zero-time controls.

To verify that the chain lengths of the products were those of the corresponding substrates, the ether-extracted products (with an unlabeled free fatty acid mixture as carrier) were neutralized to phenolphthalein endpoint with KOH and then derivatized with bromphenacyl bromide and chromatographed by reverse-phase HPLC. A C18 column was used in conjunction with an acetonitrile/water gradient.

In all cases, only one chain length of product was observed, identical to the substrate chain length.

Example 11B

The preparation from Example 9C is relatively selective in its hydrolysis of acyl-ACP thioesters, as shown in the following table: (These activities were determined as follows. Twenty five  $\mu$ l of sample were added to 75 $\mu$ l assay buffer comprising 100mM glycine-KOH pH 9, 0.2% (w/v) CHAPS, 10mM 2-mercaptoethanol, and containing radiolabeled acyl-ACP for a final concentration of 0.5 $\mu$ M. After incubation at 30°C for 60 min, the reaction was terminated by addition of 0.5ml 10% (v/v) acetic acid, and the liberated fatty acid product was extracted with 1ml diethyl ether. Enzyme activity was measured by the radioactivity of this ether extract, determined by liquid scintillation counting. A correction was applied for the small amount of non-enzymatic hydrolysis that took place.)

Table 7

	<u>Substrate</u>	<u>Activity (cpm)</u>
20	10:0-ACP	1010
	12:0-ACP	393
	14:0-ACP	30
	16:0-ACP	262
	18:1-ACP	696

25

The removal of long-chain thioesterase is incomplete, as evidenced by the partial overlap of all peaks from the Blue 4 Agarose column, and the data shown in the above table.

Example 12 - C-18 Preferring Acyl-ACP Thioesterase Assay

To assay for long chain thioesterase activity 10 $\mu$ l of the enzyme source to be analyzed is incubated at room temperature for 10 minutes in a solution comprising 100mM Tricine-NaOH, pH 8.5, and 3 $\mu$ M  $^{14}$ C stearoyl-ACP or 3 $\mu$ M  $^{14}$ C oleoyl-ACP, in a total volume of 50 $\mu$ l. Acyl-ACP substrates are prepared as described in Example 1 for preparation of lauroyl-ACP and radiolabeled in the carboxylate group at a specific radioactivity of 50-60 $\mu$ Ci/ $\mu$ mol.

The reaction is stopped by the addition of 50 $\mu$ l H<sub>2</sub>O and 100 $\mu$ l isopropanol which contains 1mM each of stearic acid and oleic acid. The fatty acid product of the hydrolytic enzyme action is extracted away from the unhydrolyzed substrate by adding 1ml petroleum ether that is saturated with 50% isopropanol in H<sub>2</sub>O. After settling for a few minutes, an aliquot of the petroleum ether layer is removed for determination of radioactivity by liquid scintillation spectrometry.

20      Example 13 - Safflower C-18 Preferring Acyl-ACP Thioesterase Purification and Identification

An initial purification of thioesterase protein from developing safflower seeds which initially follows the method of McKeon and Stumpf (J. Biol. Chem. (1982) 257:12141-12147), is described. Developing safflower seeds from greenhouse grown plants are harvested between 16 and 18 days after anthesis, frozen in liquid nitrogen and stored at -70°C.

Approximately 50g of frozen seeds are ground in liquid nitrogen and sieved to remove large seed coat pieces to yield a fine powder. The powder is washed with acetone on a Buchner funnel until all yellow color is absent from the 5 filtrate. The powder is then air dried and further processed as described below, or may be stored frozen at -70°C.

The dried acetone powder is weighed and triturated with fifteen times its weight of 20mM potassium phosphate, 10 pH 6.8. The mixture is then centrifuged at 12,000 x g for 20 minutes and decanted through a layer of Miracloth.

The acetone powder extract is acidified with glacial acetic acid to pH 5.2, held on ice for 30 minutes, and then centrifuged at 12,000 x g for 10 minutes. The supernatant 15 is adjusted to pH 4.4 with glacial acetic acid, held on ice for 30 minutes, and then centrifuged as above. The precipitate is resuspended 0.02M potassium phosphate (pH 6.8), the pH is adjusted to 6.8, and the suspension is clarified by centrifugation at 12,000 x g for 10 minutes.

20 A column of ACP-Sepharose 4B for affinity chromatography is prepared as described in Example 2. ACP is isolated from *E. coli* strain K-12 as described in Example 1. The clarified supernatant from the acid precipitation is dissolved in ACP column buffer (20mM potassium phosphate, 25% glycerol (w/v), 0.1% CHAPS (w/v), 25 pH 6.8) and applied to a 2.5cm x 3.7cm ACP-Sepharose 4B column at an application rate of 50ml per hour. The activity is eluted with a 5 bed volume gradient of 20-400mM potassium phosphate in ACP column buffer. The activity

eluted in a single peak at 180-320mM potassium phosphate, and recovery of the thioesterase was 100%.

The active fractions from the above ACP-Sephadex column were pooled, diluted to 20mM potassium phosphate,

5 0.1% CHAPS, 25% glycerol, and applied to a different ACP-Sephadex column and chromatographed as described above.

The resulting material was then applied to a chromatofocusing column for further purification of safflower thioesterase activity. The buffer of the "flow-through" from the second ACP column was changed to 20mM bis-Tris-HCl, 25% glycerol (w/v), 0.1% CHAPS (w/v), pH 7.4, ("Start" buffer) by concentration and dilution in an Amicon (Danvers, MA) stirred-cell ultrafiltration apparatus using a PM-10 membrane. A chromatofocusing column (1cm x 15 7.3cm) is packed with Pharmacia PBE 94 which has been equilibrated in "Start" buffer. The sample is applied to the PBE 94 column at a rate of 35ml per hour and the column is washed with 3 bed volumes of start buffer. The pH gradient is formed and the protein is eluted at 60ml per 20 hour by the application of 82ml of elution buffer which contains, per 100ml, 10ml Pharmacia PB 74, 25g glycerol, 1g CHAPS, and enough HCl to reach pH 4.0. An additional two bed volumes of the elution buffer is applied after the pH of the column has reached 4.0. The safflower thioesterase 25 activity elutes in two peaks, one at about pH 5.2, and the second peak spanning from pH 4.5 to 4.0. Fractions representing these activity peaks are analyzed by SDS-PAGE (Laemmli, *supra*) and silver staining.

In both peaks, two major bands were observed which correlate with thioesterase activity. These bands represent proteins having relative molecular weights of 34 and 40 kD as estimated by comparison to protein standards.

- 5       The fractions of the two activity peaks from the chromatofocusing are pooled separately, and concentrated as described above. The 34 and 40 kD thioesterase proteins are further isolated for amino acid sequencing by transfer of these proteins to either nitrocellulose or PVDF (either  
10      Immobilon-P (Millipore; Bedford, MA) or ProBlott (Applied Biosystems; Foster City, CA)) membranes following SDS-PAGE. Nitrocellulose is preferred when proteins will be subsequently enzymatically digested, while ProBlott is preferred for N-terminal sequencing methods and Immobilon-P  
15      for samples to undergo cyanogen bromide digestion.

Example 14 - Plant Thioesterase Sequencing

In this example, amino acid and nucleic acid sequencing of two exemplified plant acyl-ACP thioesterases  
20      is described. This technique may also be employed for the sequencing of other plant thioesterases of this invention as well.

All sequencing is performed by Edman degradation on an Applied Biosystems 477A Pulsed-Liquid Phase Protein  
25      Sequencer; phenylthiohydantoin (PTH) amino acids produced by the sequencer are analyzed by an on-line Applied Biosystems 120A PTH Analyzer. Data are collected and stored using an Applied BioSystems model 610A data analysis system for the Apple Macintosh and also on to a Digital

Microvax using ACCESS\*CHROM software from PE NELSON, Inc. (Cupertino, CA). Sequence data is read from a chart recorder, which receives input from the PTH Analyzer, and is confirmed using quantitative data obtained from the 5 model 610A software. All sequence data is read independently by two operators with the aid of the data analysis system.

For peptide samples obtained as peaks off of an HPLC, the sample is loaded onto a Polybrene coated glass fiber 10 filter (Applied Biosystems, Foster City, CA) which has been subjected to 3 pre-cycles in the sequencer. For peptides which have been reduced and alkylated, a portion of the PTH-amino acid product material from each sequencer cycle is counted in a liquid scintillation counter. For protein 15 samples which have been electroblotted to Immobilon-P, the band of interest is cut out and then placed above a Polybrene coated glass fiber filter, pre-cycled as above and the reaction cartridge is assembled according to manufacturer's specifications. For protein samples which 20 have been electroblotted to ProBlott, the glass fiber filter is not required.

In order to obtain protein sequences from small amounts of sample (5-30 pmoles), the 477A conversion cycle and the 120A analyzer as described by Tempst and Riviere 25 (*Anal. Biochem.* (1989) 183:290).

#### A. Sequencing of Proteolytic Fragments

A sample of Bay thioesterase purified through the ACP-Sepharose step of Example 2 is prepared for proteolytic digestion and sequencing. The sample (12 $\mu$ g of thioesterase

in 80 $\mu$ l) is denatured and reduced by heating to 95°C for 5 minutes in 160 $\mu$ l of Andersons' sample buffer (Anderson & Anderson, *Anal. Biochem.* (1978) 85:331-340) containing 2% sodium dodecyl sulfate, 5%  $\beta$ -mercaptoethanol, 20% glycerol,  
5 2% 3/10 ampholytes, and 2% Triton X-100. Proteins in 20 $\mu$ l aliquots (1 $\mu$ g total protein in each) are separated by two-dimensional electrophoresis as described by Anderson and Anderson (*Anal. Biochem.* (1978) 85:331-340 and 341-354), except that the second dimension slab gel is 1.5mm in  
10 thickness. After the second dimension electrophoresis, each of the slab gels is removed and proteins within the gel are blotted directly to a nitrocellulose membrane in a transblot system (Bio-Rad, Richmond, CA) using the method of Towbin et al (*Proc. Nat. Acad. Sci. USA* (1979) 76:4350-  
15 4354). The protein spots on the membrane are detected by reversible staining with Ponceau S (Sigma, St. Louis, MO) as described by Salinovich and Montelaro (*Anal. Biochem.* (1986) 156:341-347). Alternatively the spots may be detected by staining with amidoblack (Schaffner and  
20 Weissman, *Anal. Biochem.* (1973) 56:502-514).

For preparations of Bay thioesterase or of thioesterases having undergone an additional chromatographic purification step, one-dimensional polyacrylamide gel electrophoresis is sufficient to produce  
25 protein pure enough for sequencing. In this case, the sample (12 $\mu$ g of thioesterase in 80 $\mu$ l) is reduced and denatured by heating to 95°C for 5 min with 20 $\mu$ l of a sample buffer containing 25% (v/v) glycerol, 2.5% (w/v) sodium dodecyl sulfate (SDS), and 25% (v/v)  $\beta$ -

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mercaptoethanol in 0.156M Tris-HCl, pH 6.8. Proteins in separate aliquots (30-35 $\mu$ l) of the sample are separated by one-dimensional electrophoresis as described by Laemmli (*Nature* (1970) 227:680-685), one aliquot per 1-cm lane on a 5 1.5mm thick gel. After completion of the electrophoresis, the gel is removed, blotted, and thereafter the samples are treated as described for the two-dimensional case.

In preparation for digestion, spots corresponding to thioesterase protein are cut out of each of the membrane 10 blots and are pooled together in a plastic test tube. The methods of treatment and digestion have been described by Aebersold et al (*Proc. Nat'l Acad Sci. U.S.A.* 84:6970-6974)). The membrane pieces are treated for 30 min at 37° C with 1.0-1.2ml of freshly prepared 0.5% (w/v) 15 polyvinylpyrrolidone with average molecular weight of 40,000 (PVP-40, Aldrich, Milwaukee, WI) dissolved in 100mM acetic acid. The excess PVP-40 is removed by several washes with 3-4ml of water (HPLC grade), removal of PVP-40 is complete when the absorbance at 214nm of successive 20 washes no longer decreases or reaches that of a water blank. The pieces are then removed from the wash tube, minced and are placed in a 1-ml Eppendorf plastic tube, and 100mM Tris-HCl or 100mM sodium carbonate, pH 8.2/acetonitrile, 95:5 (v/v) is added so that the liquid 25 just covers the top of them. The digest is started by addition of 10 $\mu$ l of Boehringer Mannheim sequence grade trypsin (100 $\mu$ g/ml solution in 1% HCl), and the sample is allowed to digest at 37°C for 8-24 hr., with occasional stirring. The amount of protease added is usually between

1/20 and 1/10 of the weight of protein being digested.

Peptides elute from the membrane into the digest buffer during the incubation. The digestion is terminated by addition of 10 $\mu$ l of 10% (v/v) trifluoroacetic acid (TFA).

5 Alternatively the chips may be suspended in 100mM sodium phosphate or 25mM ammonium carbonate, pH 7.8/acetonitrile, 95:5 (vo/v), and digested for 8-24 hours at 25°C with 10 $\mu$ l of Boehringer Mannheim sequence grade endoproteinase gluC (100 $\mu$ g/ml solution in water).

10 Digestion with trypsin allows cleavage at lysine and arginine residues, whereas digestion with gluC cleaves at glutamic acid residues (and also aspartic acid under some conditions) depending upon the buffer. Digestion of separate samples with each of the proteases affords 15 identification of overlapping peptides and construction of longer peptide sequences useful for PCR technology.

The digest mixture is removed from the nitrocellulose pieces, the nitrocellulose pieces are washed with 1-5 100 $\mu$ l volumes of 0.05% (v/v) TFA, and these volumes are 20 concentrated to a volume of less than 100 $\mu$ l in a Speed-Vac (Savant; Farmingdale, NY). These concentrates are then injected over a Vydac reverse phase Protein & Peptide C18 column (2.1mm x 100mm) installed in an Applied Biosystems (Foster City, CA) Model 130 High Performance Liquid 25 Chromatograph (HPLC). Mobile phases used to elute peptides were: Buffer A: 0.1mM sodium phosphate, pH2.2; Buffer B: 70% acetonitrile in 0.1mM sodium phosphate, pH2.2. A 3-step gradient of 10-55% buffer B over two hours, 55-75% buffer B over 5 minutes, and 75% buffer B isocratic for 15

minutes at a flow rate of 50 $\mu$ l/minute is used. Peptides are detected at 214nm, collected by hand, and then stored at -20° C.

Separation of the released peptides may also be  
5 accomplished through reverse-phase HPLC on a C18 (2 x 150mm) column using a 120-min gradient increasing from 7% to 70% acetonitrile in 0.1% TFA at a flow rate of 50 $\mu$ l per min. The elution of peptides is monitored by absorbance at 214nm, each peptide being collected into a separate  
10 fraction tube. The peptides are stored frozen at -20°C until application to the protein sequencer (Applied Biosystems, Foster City, CA).

Alternatively, the peptides may be alkylated before separation on HPLC. Alkylation allows identification of  
15 cystine residues on the sequencer, which otherwise go undetected. The unacidified digest mixture is reduced by addition of 1 $\mu$ l of 10% (v/v)  $\beta$ -mercaptoethanol (1.43 $\mu$ mol) and incubated at 37°C for 2 hours. The reduced peptides are then alkylated with approximately 1.6 $\mu$ mol of [<sup>3</sup>H]-  
20 iodoacetic acid (200mCi/mmol) for 30 min at room temperature in the dark. Depending upon the concentration of  $\beta$ -mercaptoethanol the [<sup>3</sup>H]-iodoacetic acid may be adjusted to a ratio of 1:1.1. The mixture is then acidified with 10 $\mu$ l of 10% (v/v) TFA, applied to the  
25 reverse-phase HPLC column and further treated as described above. Other alkylating agents may be used including iodoacetamide and 4-vinylpyridine. The latter reagent leads to formation of pyridylethyl-cysteine residues which are identifiable on the protein sequencer by the unique

retention time of its corresponding PTH-amino acid derivative.

The Bay thioesterases of the 34 kD doublet are sequenced independently (A and B). Peptide sequences are 5 shown in Table 8. It is noted that several of the band A and B peptides were either identical or near identical in sequence.

Table 8

10		<u>BAND "A"</u>	
SQ 736	SEQ ID NO: 1	YPTWPNFVL-T(M) L (I) (G) (A) (Q)	
SQ 737	SEQ ID NO: 2	DLMWVV	
SQ 739	SEQ ID NO: 3	-GYNP- (D) IPFVG I	
15	SQ 740	LND-- (HPLC crashed after #3)	
SQ 741	SEQ ID NO: 4	(T)-TLVDVV(P)FVIWFVFIDNVAVK	
SQ 742	SEQ ID NO: 5	LNDLTADYIQS-LTP (R) S G	
SQ 743	SEQ ID NO: 6	AG (G) WVFETVPDXIFE	
20	SQ 745	SEQ ID NO: 7	NETGVIFVVMVV (A) FGP (I) K I
SQ 747	SEQ ID NO: 8	SVGILGDGFGTLEMSK G	
SQ 749	SEQ ID NO: 9	GISVIPAEP (R)	

"BAND B"

	SQ 696	SEQ ID NO: 10	LNDSTADYIQGGLTP L
5	SQ 697	SEQ ID NO: 11	SVGILGDGFGTTLXMSK
	SQ 698	SEQ ID NO: 12	GISVIPAEPR
	SQ 699	SEQ ID NO: 13	YVA (E) VFETVPDXIF
	SQ 701	SEQ ID NO: 14	STDILAVMNXMQFATLNXAK
	SQ 702	SEQ ID NO: 15	--IGPAF (I) DNVAVK
10	SQ 703	SEQ ID NO: 16	--IGPAFIDNVAVK
	SQ 704	SEQ ID NO: 17	(S) TSLSVLMNT
	SQ 765	SEQ ID NO: 18	DSIFES
	SQ 766	SEQ ID NO: 19	DYIQGGLTP-W
	SQ 767	SEQ ID NO: 20	DSVL-SLTTV-GGSSEA
15	SQ 768	SEQ ID NO: 21	DTVEVE-IIANs S
	SQ 769	SEQ ID NO: 22	D-FrGISVIPAEPr
	SQ 770	SEQ ID NO: 23	DSFrGISIVAEPr
	SQ 772	SEQ ID NO: 24	DWVIEYrPGV
20	SQ 773	SEQ ID NO: 25	DHLLeLEGGsEVL-a

N-terminal proteins can also be sequenced without digestion. For example, proteins are electroblotted to Immobilon-P PVDF for 30 minutes in the following buffer:

25 12.5mM Tris/5mM glycine in 10% (v/v) methanol. Following electroblotting, membranes are stained in 0.1% (w/v) Coomassie Blue in 50% (v/v) methanol/10% (v/v) acetic acid for 5 minutes and destained in 2-3 changes of 50% (v/v) methanol/10% (v/v) acetic acid, 2 minutes for each change.

30 Following this, PVDF membranes are allowed to air dry for

30 minutes and are then stored dry in heat-sealed plastic bags at -20°C. Protein blotted to PVDF is used directly to determine N-terminal sequence of the intact protein.

In this manner, the N-terminal amino acid of the bay

5 34 kD thioesterase of band B is determined as:

SQ 837 SEQ ID NO: 39 LEWKPKPK(L)PE(L)LD

Further, the sequence of a bay thioesterase which migrates slightly faster than the Band B peptide is determined as:

10 SQ 840 SEQ ID NO: 40 LLDDHFGLHGLVFRRTFAIRSYEVGPDR

B. Cyanogen Bromide Cleavage of Protein and Separation of Peptides

As an alternative method cyanogen bromide cleavage may 15 be performed. For example, as exemplified with the 34 and 40 kD safflower thioesterase proteins using the methodology described in the Probe-Design Peptide Separation System Technical Manual from Promega, Inc. (Madison, WI). The peptides shown below in Table 9 were obtained in this 20 manner.

The proteins are electrophoresed on SDS-polyacrylamide gels (Laemmli, *supra*) and blotted to Immobilon-P PVDF membrane as described above. Protein bands are cut out of the blot and each band is placed in a 1.5ml microcentrifuge 25 tube containing 200 $\mu$ l of a 10mg/ml solution of cyanogen bromide in 70% (v/v) formic acid. Protein bands are incubated in this solution overnight at room temperature, and following this incubation the cyanogen bromide solutions are removed and pooled. The pooled solution is

- dried under a continuous nitrogen stream using a Reacti-Vap Evaporator (Pierce, Rockford, IL). Cyanogen bromide peptides are eluted off the Immobilon-P PVDF membrane using a peptide elution solvent with the following composition:
- 5 70% (v/v) isopropanol, 0.2% (v/v) trifluoroacetic acid, 0.1mM lysine, and 0.1mM thioglycolic acid. 200 $\mu$ l of this elution solvent is added to each tube and tubes are incubated for 2 hours at room temperature with occasional vortexing. The elution solvents are then removed from each 10 tube, pooled, added to the tube containing the dried cyanogen bromide solution, and dried as described above. The elution procedure is repeated with fresh elution solvent for an additional 2 hours and the pooled solvent is added to the previously dried material and again dried.
- 15 50 $\mu$ l of HPLC grade water is then added to the dried peptides and the water removed by evaporation in a Speed-Vac (Savant, Inc., Farmingdale, NY).
- Peptides are separated using a Tris/Tricine SDS-PAGE system similar to that described by Schägger and von Jagow 20 (Anal. Biochem. (1987) 166:368-379). Either 16% or 10-20% (gradient) acrylamide tricine-SDS-PAGE pre-cast gels (Novex Inc., Encinitas, CA), are used for the separation. Gels are run in a Tall Mighty Small electrophoresis apparatus from Hoefer Scientific Instruments (San Francisco, CA).
- 25 Prior to electrophoresis of the peptides, gels are pre-run with thioglycolic acid added to the cathode buffer at a concentration of 0.1-0.2mM for 30-60 minutes at a constant voltage of 30 volts. Running buffer used is made up from a 10X stock, also from Novex; final concentration (1X) is

0.1M Tris, 0.1M Tricine and 0.1% (w/v) SDS. The dried peptides are resuspended in 15 $\mu$ l HPLC grade water and 15 $\mu$ l 2X sample buffer consisting of: 0.125M Tris-HCl, 2% (w/v) SDS, 5% (v/v)  $\beta$ -mercaptoethanol, 20% (v/v) glycerol, and 5 0.0025% (w/v) bromphenol blue, and boiled for 5 minutes prior to loading on the gel.

Gels are run at a constant voltage of 125-150 volts for approximately 1 hour or until the tracking dye has begun to run off the bottom edge of the gel. Gels are 10 soaked in transfer buffer (125mM Tris, 50mM glycine, 10% (v/v) methanol) for 15-30 minutes prior to transfer. Gels are blotted to ProBlott sequencing membranes for 2 hours at a constant voltage of 50 volts. The membranes are stained with Coomassie blue (0.1% in 50% (v/v) methanol/10% (v/v) 15 acetic acid) and destained for 3X 2 min. in 50% (v/v) methanol/10% (v/v) acetic acid. Membranes are air-dried for 30-45 minutes before storing dry at -20°C.

Peptides blotted on to ProBlott can be directly loaded to the sequencer cartridge of the protein sequencer without 20 the addition of a Polybrene-coated glass fibre filter. Peptides are sequenced using a slightly modified reaction cycle, BLOT-1, supplied by Applied Biosystems. Also, solution S3 (butyl chloride), is replaced by a 50:50 mix of S1 and S2 (n-heptane and ethyl acetate). These two 25 modifications are used whenever samples blotted to ProBlott are sequenced.

Amino acid sequences of cyanogen bromide fragments of the 34 and 40 kD proteins are determined by the N-terminal sequencing method described above. Sequences obtained in

this manner are presented in Table 9, wherein the one-letter abbreviation for amino acids is used and X indicates an unidentified amino acid.

5

Table 9

S828	SEQ ID NO: 26	GSLTEDGLSYKEVFIIRXYEVGINKTA
S829	SEQ ID NO: 27	NKHNNTYIGXVLESIPQEVIDTHELQTITLDYRRE
S830	SEQ ID NO: 28	AVRTGEQPTGVAVGLKEA
10	S833 SEQ ID NO: 29	KDHASGQVIG
S834	SEQ ID NO: 30	NEDTRRLQKVNDDEYLVFIP
	S834B SEQ ID NO: 31	HIEIYXYP

As the above protocol results in partial cyanogen bromide cleavage, peptides of varying relative molecular weights having common amino acid sequences are obtained. The amino acid sequence of one peptide (sequence not shown) was determined to correspond with amino acid sequence of a safflower desaturase protein (Thompson et al., *Proc. Nat. Acad. Sci. (1991) 88:2578-2582*).

#### C. Isolation and Assembly of cDNA

Once partial amino acid sequences are determined, they may be used to obtain DNA sequence of the plant thioesterase via Polymerase Chain Reaction (PCR) technology. Thus, oligonucleotide fragments are synthesized on an Applied Biosystems model 380A DNA synthesizer to amino acid sequences which have the least redundancy for use as PCR primers. Restriction sites are designed into the ends of the oligonucleotide primers so

that the resulting DNA fragments may be readily manipulated in cloning. Purified genomic DNA or RNA isolated from the plant thioesterase source are used as templates in reaction.

5 PCR reactions are run using Taq polymerase (Gene Amp Kit) and the DNA thermal cycler (Perkin-Elmer/Cetus) in two different combinations of the oligonucleotides as 5'- or 3'- primers. The resulting DNA products are run on an agarose gel for separation. DNA sequences are determined  
10 by the dideoxy-chain termination method of Sanger et. al., *Proc. Natl. Acad. Sci. USA* (1977) 74:5463-5467) using the 7-Deaza-dGTP Reagent Kit with Sequenase Version 2 Enzyme (United States Biochemical Corp., Cleveland, OH). The sequence data are analyzed using the IntelliGenetics Suite  
15 of molecular programs Gel and SEQ.

#### 1. RNA Isolation

Total RNA is isolated from developing Bay seeds according to the method of Turpen and Griffith (*Biotechniques* (1986) 4:11-15). Briefly, 50g of fresh  
20 frozen material is homogenized in 4M guanidine thiocyanate and 2% sarcosyl. The cleared lysate is layered upon a 5.7M CsCl cushion and centrifuged for 5.5 hours at 50,000 rpm. The RNA pellet is dissolved in water, extracted with phenol and chloroform, and precipitated with ethanol. The  
25 resulting pellet is resuspended in water and represents the total RNA fraction. Poly (A) RNA is isolated from this material according to Maniatis et al. (*Molecular Cloning: A Laboratory Manual* (1982) Cold Springs Harbor, New York).

2. PCR Generation of a Partial Thioesterase cDNA

The protein sequence information from the peptides of Table 8 is used to design degenerate oligonucleotides (SEQ ID NO: 32-33). (See, Fig. 1). These oligonucleotides are 5 used as primers in order to amplify thioesterase sequence from Bay embryo total cDNA (Lee et al. *Science* (1988) 339:1288-1291). Thus, poly (A) RNA from Bay embryos is reverse transcribed with M-MLV reverse transcriptase (BRL, Bethesda, MD) to obtain a single strand cDNA. This cDNA is 10 used as a template for the thioesterase specific oligonucleotides in a PCR. The reaction is carried out according to manufacturer's instructions having the thermal cycler set for the following cycling program: 30 cycles; 1 min. at 94°, 1 sec. at 65°, slope of 2 min from 65° down to 15 50°, and 2 min. at 74°. PCR reactions are analyzed by agarose gel electrophoresis. The DNA fragment corresponding to the resulting 800 bp band is cloned. DNA sequence analysis (SEQ ID NOS: 34-35 and Fig. 2) shows that indeed this DNA fragment codes for several of our 20 thioesterase peptides.

3. Isolation of Thioesterase cDNA Clones.

The 800 bp PCR-generated DNA fragment is labeled with <sup>32</sup>P (Random Primed DNA labelling Kit, Boehringer Mannheim, Indianapolis, IN) and used as a probe to screen 25 approximately 2 million plaques of a conventionally created cDNA library: (double stranded, oligo dT primed cDNA is synthesized from the Bay seed poly(A) RNA according to Gubler and Hoffman, *Gene* (1983) 25:263-269; EcoRI linkers are ligated to the ends, and the resulting material cloned

into a bacteriophage expression vector, LambdaZAP,  
Stratagene; La Jolla, CA.

The longest library clone overlaps for 112 bp with our  
PCR sequence (100% sequence match in this stretch). It  
5 extends further to the 3' end of the transcript, see Fig.  
2.

By linking the 800 bp PCR fragment with the longest  
bacteriophage clone at the shared *Hind*III site (See, Fig.  
2, lane (345)), a 1200 bp long contiguous DNA fragment with  
10 a potential reading frame of about 1000 coding basepairs is  
created.

To obtain the full clone, a second cDNA library may be  
constructed from bay poly(A)+ RNA in the plasmid cloning  
vector pCGN1703. pCGN1703 is derived from the commercial  
15 cloning vector Bluescribe M13- (Stratagene Cloning Systems;  
San Diego, CA), and made as follows. The polylinker of  
Bluescribe M13- is altered by digestion with *Bam*HI,  
treatment with mung bean endonuclease, and blunt-end  
ligation to create a *Bam*HI-deleted plasmid, pCGN1700.  
20 pCGN1700 is digested with *Eco*RI and *Sst*I (adjacent  
restriction sites) and annealed with a synthetic linker  
having restriction sites for *Bam*HI, *Pst*I, *Xba*I, *Apa*I and  
*Sma*I, a 5' overhang of AATT, and a 3' overhang of TCGA.  
The insertion of the linker into pCGN1700 eliminates the  
25 *Eco*RI site, recreates the *Sst*I (also, sometimes referred to  
as "SacI" herein) site found in Bluescribe, and adds the  
new restriction sites contained on the linker. The  
resulting plasmid pCGN1702, is digested with *Hind*III and  
blunt-ended with Klenow enzyme; the linear DNA is partially

digested with *Pvu*II and ligated with T4 DNA ligase in dilute solution. A transformant having the *lac* promoter region deleted is selected (pCGN1703) and is used as the plasmid cloning vector.

- 5        Briefly, the cDNA synthesis is as follows. The plasmid cloning vector is digested with *Sst*I and homopolymer T-tails are generated on the resulting 3'-overhang stick-ends using terminal deoxynucleotidyl transferase. The tailed plasmid is separated from  
10 undigested or un-tailed plasmid by oligo(dA)-cellulose chromatography. The resultant vector serves as the primer for synthesis of cDNA first strands covalently attached to either end of the vector plasmid. The cDNA-mRNA-vector complexes are treated with terminal transferase in the  
15 presence of deoxyguanosine triphosphate, generating G-tails at the ends of the cDNA strands. The extra cDNA-mRNA complex, adjacent to the *Bam*HI site, is removed by *Bam*HI digestion, leaving a cDNA-mRNA-vector complex with a *Bam*HI stick-end at one end and a G-tail at the other. This  
20 complex is cyclized using an annealed synthetic cyclizing linker which has a 5' *Bam*HI sticky-end, recognition sequences for restriction enzymes *Not*I, *Eco*RI and *Sst*I, and a 3' C-tail end. Following ligation and repair the circular complexes are transformed into *E. coli* strain DH5 $\alpha$   
25 (BRL, Gaithersburg, MD) to generate the cDNA library. The bay embryo cDNA bank in plasmid vector pCGN1703 contains approximately  $1.5 \times 10^6$  clones with an average cDNA insert size of approximately 500 base pairs.

A full length cDNA of the bay thioesterase, 3A-17 (pCGN3822), was isolated from the pCGN1703 library by screening with the  $^{32}\text{P}$ -labeled 800 bp PCR-generated fragment of thioesterase as described above.

5        4. cDNA Sequence

In summary, approximately 1200 bp of contiguous DNA sequence is shown in Fig. 2. This comprises about 80-90% of the coding region for the mature Bay thioesterase and a 200 bp 3' untranslated sequence containing translational stop 10 and poly(A) addition sequences.

The 580 bp of coding region now sequenced is estimated to be about 60% of the total coding frame of the mature protein. This partial sequence, when translated, codes for a polypeptide which contains many sequences from Table 8 15 (SEQ ID NOS: 1-25), some are shown aligned in Fig. 2.

Peptides not coded for might be located in the not yet sequenced regions of the cDNA's or come from entirely different proteins. Several other peptides, like peptide 701 (SEQ ID NO: 14), are slightly different from the 20 predicted protein sequence, see Fig. 2. This may indicate the presence of a gene family for the thioesterase.

A second 580 bp DNA fragment obtained through the cDNA library screen may also provide evidence of a gene family. This sequence shows approximately 80% sequence identity 25 with the clone at the DNA level described above (Fig. 3). The sequence in the upper line (SEQ ID NO: 35) represents the clone described above and the lower sequence line (SEQ ID NO: 36) represents the second 580 bp fragment fragment. At the amino acid level more degeneracy is seen.

Sequence (SEQ ID NO: 38) of the full length thioesterase cDNA clone, 3A-17, is presented in Fig. 4B. The translated sequence (SEQ ID NO: 37) of this clone is presented in Fig. 4A. The exact "start" methionine has not been determined, but is one of the methionines located at positions 1, 5 and 13 of the translated amino acid sequence.

10       Example 15 - Isolation of a Safflower C-18 Preferring Acyl-ACP Thioesterase cDNA

Sequence information from cyanogen bromide peptide sequences (SEQ ID NOS: 26-31) of the safflower 34 and 40 kD protein bands of Table 9 from Example 14B is analyzed to obtain a peptide map of the safflower thioesterase protein.

15       Comparison of the molecular masses (as estimated by SDS-PAGE) of peptides having common amino acid sequences is used to determine the order and distance between these peptides in the thioesterase protein. Homology comparisons of these peptides to the amino acid sequence of the bay thioesterase (Fig. 4B) confirms the peptide map shown in Fig. 5. Numbers between peptide sites indicate estimated base pair separation on a thioesterase cDNA for sequences which correspond to the S828, S829, S830 and S834 peptide sequences.

20       Degenerate oligonucleotide primers for PCR are designed from amino acid sequences of safflower thioesterase peptide fragments S828, S829, S830 and S834. The S830-derived oligonucleotide mixture, 830, is used as forward primer (binds to antisense strand and primes

25       synthesis of sense thioesterase DNA) and the S829

oligonucleotide mixtures, 829-1R and 829-2R, are used as reverse primers (bind to sense strand and prime synthesis of antisense thioesterase DNA) in PCR reactions utilizing safflower seed cDNA (from cDNA library described below) as template.

Oligonucleotide mixture 830 contains all possible sequences that could encode amino acids 5-11 of peptide S830, except that the codon chosen for the glycine at position 5 is GGC, the codon ACC is chosen for the threonine at position 9 (with an inosine also being included at the third base), and only the first two nucleotides of the possible codons are included for the valine at position 11. S830 also contains non-thioesterase sequences at the 5' end which code for an NcoI site to facilitate cloning of the PCR products.

Oligonucleotide mixture 829-1R contains all possible complements of sequences that could encode amino acids 1-6 of peptide S829, except that only the first two nucleotides of the possible codons are included for the asparagine at position 6. 829-1R also contains the complement for a methionine codon at the 3' end, as it can be assumed that there is a methionine residue at that position which was cleaved in the cyanogen bromide digestion. S829-1R also contains non-thioesterase sequences at the 5' end which code for a HindIII site to facilitate cloning of the PCR products.

Oligonucleotide mixture 829-2R contains all possible complements of sequences that could encode amino acids 19-25 of peptide S829, except that an inosine base is included

for the third position of the codon for the isoleucine at position 22, the codon ACG is chosen for the threonine at position 24 (with an inosine also being included at the third base), and only the first two nucleotides of the 5 possible codons are included for the histidine at position 25. 829-2R also contains non-thioesterase sequences at the 5' end which code for a *Hind*III site to facilitate cloning of the PCR products.

Similarly, oligonucleotide mixtures are designed from 10 amino acids 10-16 of peptide S828 (828 is a forward primer with *Bam*HI site sequences at the 5' end), amino acids 12-18 of peptide S834 (834 is a forward primer with *Xba*I site sequences at the 5' end), and amino acids 8-14 of peptide S834 (834R is a reverse primer with *Sal*I site sequences at 15 the 5' end).

PCR reactions are run using *Taq* polymerase and the DNA thermo cycler (Perkin/Elmer Cetus) according to manufacturer's specifications. Cycle parameters may be altered to provide for maximum yield of the thioesterase 20 PCR product.

PCR products are analyzed by agarose gel electrophoresis and the expected ~800bp band is observed. Oligonucleotides derived from S834 and S828 are used to verify that the band represents thioesterase DNA, either by 25 further PCR using the S830/S829 PCR product as template, or by Southern hybridization of S830/S829 PCR product. DNA sequence of the ~800bp product is determined to verify that the fragment codes for a portion of the safflower thioesterase protein.

The ~800bp thioesterase fragment is labeled with  $^{32}\text{P}$  and used as a probe to screen a safflower cDNA library constructed in the plasmid cloning vector, pCGN1703. The cDNA library is constructed from poly(A)+ RNA isolated from 5 safflower embryos harvested at days 14-17 post-anthesis by a method initially described by Jackson and Larkins (*Plant Physiol.* (1976) 57:5-10) as modified by Goldberg et al. (*Developmental Biol.* (1981) 83:201-217).

The polyadenylated RNA is used to construct a cDNA 10 library in the plasmid cloning vector pCGN1703, as described in Example 14.C.3. The safflower embryo cDNA bank obtained in this manner contains between approximately  $3-5 \times 10^6$  clones with an average cDNA insert size of approximately 1000 base pairs.

15

Example 16 - Expression of Medium-Chain Preferring Acyl-ACP Thioesterase In *E. coli*

In this example, expression of bay thioesterase proteins in *E. coli* is described.

20 The truncated Bay (1200 bp) cDNA described in Example 14 is expressed as a 30 kD protein in an *E. coli* host cell and data is provided demonstrating that the cDNA fragment confers upon the transformant an increased C12 acyl-ACP thioesterase activity.

25 A pET3a vector (Rosenberg, et al., *Gene* (1987) 56:125-135) is used in an *E. coli* strain BL21 (DE3) (Studier and Moffat, *J. Mol. Biol.* (1986) 189:113-130) host for this study. The pET3a vector contains a promoter and 33 bp of the 5' reading frame of bacteriophage T7. T7 polymerase is

under the regulatory control of an isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG)-inducible lac UV5 promoter found in the *E. coli* BL21 (DE3) strain. Thus, by the addition of IPTG to *E. coli* BL21 (DE3) transformed with 5 pET3a, the T7 promoter will be activated.

Constructs are prepared containing the truncated cDNA of Fig. 2 fused in reading frame by deletion of the BamHI/EcoRI fragment and replacement of the thioesterase sequence. *E. coli* are transformed with pET3a constructs 10 containing the thioesterase (pET3a-THI0) and unmodified pET3a as a control. The *E. coli* are grown at 37°C in liquid medium and expression is induced by the addition of 1mM IPTG. After 1 hour induction, cells are harvested by centrifugation, resuspended in assay buffer and lysed by 15 sonication. Cell debris is removed by further centrifugation and the supernant used in activity assays as per Example 1.

Table 10

			Hydrolysis Activity (mean cpm in ether extract)
20	<i>E. coli</i> Lysate	Assay Substrate	
	pET3a	8:0-ACP	370
	"	10:0-ACP	787
	"	12:0-ACP	1028
25	"	14:0-ACP	1271
	"	16:0-ACP	2848
	"	18:1-ACP	2877
	pET3a-THI0	8:0-ACP	349
30	"	10:0-ACP	621
	"	12:0-ACP	2127
	"	14:0-ACP	1035
	"	16:0-ACP	1900
	"	18:1-ACP	2025

The results demonstrate that a lysate of control *E. coli* cells contains hydrolytic activity towards all the acyl-ACP substrates that were tested, with preference for the long-chain substrates. Comparing the pET3a-THIO 5 results with the control results it is evident that the pattern of substrate preferences differs. The transformant lysate shows greatly increased activity with 12:0-ACP in relation to the other substrates, as compared with the control lysate. This increased 12:0-ACP activity 10 demonstrates that this cDNA fragment comprises sufficient of the Bay 12:0-ACP thioesterase gene to produce active enzyme in *E. coli* cells.

In addition, the entire mature bay thioesterase protein is expressed as a lac fusion in *E. coli* cells. 15 Sequence analysis of the full length bay thioesterase cDNA, pCGN3822, described in Example 14, reveals an *Xba*I site at base 394. Digestion at this *Xba*I site cleaves the coding region immediately 5' of the codon representing the leucine at amino acid position 72. This leucine has been 20 identified as a candidate for the amino terminal residue as described in Example 14A.

An approximately 1200 bp fragment of pCGN3822 cDNA is generated by digestion with *Xba*I, which cuts at the postulated mature protein start site, as described above, 25 and in the vector sequences flanking the 3' end of the cDNA. The *Xba*I fragment is cloned on *Xba*I digest of the minus version of a Bluescribe M13(+/−) (also called pBS+/−) cloning vector (Stratagene; San Diego, CA.). The thioesterase gene clone is inserted such that the mature

protein is in reading frame with a portion of the lacZ gene of the Bluescribe vector and under control of the lac promoter.

- The resulting construct, pCGN3823, and a control
- 5 Bluescribe construct having the bay thioesterase gene inserted in the opposite orientation are transformed into *E. coli*. The *E. coli* cells are grown at 37°C in liquid medium and expression from the lac promoter is induced by addition of IPTG to a final concentration of 0.1mM IPTG.
- 10 Following one hour of induction, cells are harvested, lysed and assayed as described above for the truncated bay thioesterase.

Table 11

	Induced <i>E. coli</i> Lysate	Dilution	Assay Substrate	Hydrolysis Activity (mean cpm in ether extract)
15	pCGN3823	1/4000	8:0-ACP	0
	"	"	10:0-ACP	0
	"	"	12:0-ACP	1840
	"	"	14:0-ACP	116
	"	"	16:0-ACP	20
	"	"	18:1-ACP	5
20	control	1/4000	8:0-ACP	0
	"	"	10:0-ACP	0
	"	"	12:0-ACP	0
	"	"	14:0-ACP	0
	"	"	16:0-ACP	13
	"	"	18:1-ACP	6

- 30 The results demonstrate that a lysate from *E. coli* cells expressing the postulated mature bay thioesterase enzyme has significantly greater activity towards a 12:0-ACP substrate than towards other ACP substrates of varying carbon chain length. In addition, this activity is more than two orders of magnitude greater than that in a lysate of *E. coli* cells expressing the truncated bay thioesterase.

Studies are being conducted to determine if expression of the bay thioesterase protein in *E. coli* cells has an effect on the fatty acid composition of these cells. Initial studies have failed to identify a substantial change in the 5 fatty acid composites of the *E. coli* cells containing the bay thioesterase.

Substrate analysis of the bay thioesterase enzyme purified from developing seeds as described in Example 2 is also conducted. Results are presented in Table 12 below.

10

Table 12

	Assay Substrate	Hydrolysis Activity (mean cpm in)	
		Ether Extract	
15	8:0-ACP	0	
	10:0-ACP	0	
	12:0-ACP	1261	
	14:0-ACP	69	
	16:0-ACP	12	
	18:1-ACP	432	

Comparison of the results of substrate analysis of the thioesterase in the *E. coli* extracts and as purified from 25 developing bay seeds reveals that the activity profile of the enzyme from the two sources is essentially identical with respect to activity with C8, 10, 12, 14, and 16 ACP substrates. Although the enzyme purified from embryos is slightly more active with C18:1 substrates than is the *E.* 30 *coli*-expressed thioesterase, this difference is believed due to activity of a long chain bay thioesterase which is not completely removed from the medium chain thioesterase protein preparation.

Example 17 - Transformation with Plant Thioesterase

A. Constructs for expression of bay thioesterase in plant cells which utilize phaseolin, napin, CaMV35S and Bce4 promoter regions are prepared as follows.

5       Phaseolin/thioesterase

A 1.45kb fragment of 3A-17 is obtained by digestion with *Bal*I and *Sal*I. The *Bal*I site is located at position 149 of the cDNA insert, and the *Sal*I site is in the polylinker located 3' to the cDNA insert. Thus, this 10 fragment contains the entire thioesterase coding region and the entire cDNA 3' region, including the polyadenylation signal, AAATAA, located at bases 1447-1452, and also contains the restriction digestion sites *Kpn*I, *Sma*I, *Xba*I and *Sal*I located directly 3' to the cDNA.

15       An 850bp *Bgl*II fragment of the  $\beta$ -phaseolin 5' noncoding region was obtained from p8.8pro (Hoffman et al. (1987) EMBO J. 6:3213-3221) and cloned into pUC9 (Vieira and Messing, supra) at the *Bam*HI site to yield pTV796. The phaseolin fragment in pTV796 is oriented such that *Sma*I 20 site of pUC9 is located 3' to the phaseolin promoter. An ~850bp fragment is generated by digestion of pTV796 with *Hind*III and *Sma*I and gel-purified.

The phaseolin promoter (*Hind*III/*Sma*I) and thioesterase coding region (*Bal*I/*Sal*I) are joined by three way ligation 25 into a Bluescript (Stratagene) cloning vector that has been digested with *Hind*III and *Sal*I. The resulting plasmid contains the phaseolin promoter/thioesterase construct on a *Hind*III/*Sal*I fragment that is flanked by various restriction sites, including a 5' *Bam*HI site and a 3' *Kpn*I

site. No additional plant 3' noncoding region is provided as the thioesterase fragment contains a polyadenylation signal. The phaseolin promoter/thioesterase fragment may be obtained by digestion with *Bam*HI and *Kpn*I, or 5 alternatively by partial digestion with *Xba*I, and ligated into an appropriate binary vector, such as pCGN1557 or pCGN1578 (McBride and Summerfelt, (1990) *Plant Mol. Biol.* 14:269-276), for plant transformation.

35S/thioesterase/mas

10 An *Bal*I/*Pst*I fragment of the thioesterase cDNA 3A-17 containing approximately 1200bp, and including the entire coding region, is obtained by partial digestion with restriction enzymes *Bal*I and *Pst*I and gel-purification of the 1200bp fragment. The fragment is ligated into a 15 plasmid cloning vector, such as a Bluescript vector (Stratagene Cloning Systems; La Jolla, CA), that has been digested with *Pst*I and *Bam*HI, and the *Bam*HI site filled in using the Klenow fragment of DNA Polymerase I. In this procedure, the *Bam*HI site is restored by ligation to the 20 *Bal*I site of the thioesterase cDNA.

The resulting plasmid is partially digested with *Bam*HI and *Eco*RI to obtain the approximately 1200bp thioesterase fragment. This fragment is then cloned into an approximately 4.4kb *Bam*HI/*Eco*RI DNA fragment which contains 25 approximately 0.94kb of 5' noncoding sequence from a cauliflower mosaic (CaMV) 35S gene (immediately 5' to the *Bam*HI site), approximately 0.77kb of 3' noncoding sequence from an *Agrobacterium tumefaciens* manopine synthase (*mas*) gene (immediately 3' to the *Eco*RI site), and a pUC19 (New

England BioLabs, Beverly, MA) backbone. The BamHI/EcoRI DNA fragment is obtained by partial digestion of a larger plasmid vector and gel purification of the desired 4.4kb fragment. The 35S 5' region is from bases 6492 to 7433 of strain CM1841 (Gardner, et al. (1981) *Nucl. Acids Res.* 9:2871-2887), which is from about -640 to about +2 in relation to the transcription start site. The mas 3' noncoding region is from about bases 19,239 to 18,474 of octopine Ti plasmid pTiA6 (numbering corresponds to that of closely related pTi15955 as reported by Barker et al. (Plant Mol. Biol. (1983) 2:335-350)).

The resulting 35S/thioesterase/mas plasmid is digested at flanking *Bgl*III sites and cloned into a BamHI digested binary vector, such as pCGN1557 or pCGN1578 (McBride and Summerfelt, *supra*).

Bce4/thioesterase

A 1.45kb thioesterase cDNA *Bal*II/*Sal*II fragment is prepared as described above. A Bce4 expression cassette, pCGN1870, which provides for preferential expression in early seed development is described in copending US Patent Application Serial No. 07/494,722, which is incorporated herein by reference.

An approximately 1kb fragment of the Bce4 5' noncoding region whose 3' end is immediately 5' to the Bce4 start codon, is obtained by digestion of pCGN1870 with *Xba*I and *Xho*I and gel purification of the resulting 1kb fragment.

The Bce4 promoter (*Xba*I/*Xho*I) and thioesterase coding region (*Bal*II/*Sal*II) are joined by three way ligation into a Bluescribe (Stratagene) cloning vector that has been

digested with *Xba*I and *Sal*I. The resulting plasmid contains the *Bce4* promoter/thioesterase construct on a *Xba*I/*Sal*I fragment that is flanked by various restriction sites, including a 5' *Bam*HI site and a 3' *Kpn*I site. No additional plant 3' noncoding region is provided as the thioesterase fragment contains a polyadenylation signal. The *Bce4* promoter/thioesterase fragment may be obtained by digestion with *Bam*HI and partial digestion with *Kpn*I (or *Asp*718 which has the same recognition sequence), or alternatively by partial digestion with *Xba*I, and ligated into an appropriate binary vector, such as pCGN1557 or pCGN1578 (McBride and Summerfelt, *supra*), for plant transformation.

Napin/thioesterase/napin

The napin expression cassette, pCGN1808, is described in copending US Patent Application serial number 07/550,804, which is incorporated herein by reference. pCGN1808 is modified to contain flanking restriction sites to allow movement of only the expression sequences and not the antibiotic resistance marker to binary vectors such as pCGN1557 (McBride and Summerfelt, *supra*). Synthetic oligonucleotides containing *Kpn*I, *Not*I and *Hind*III restriction sites are annealed and ligated at the unique *Hind*III site of pCGN1808, such that only one *Hind*III site is recovered. The resulting plasmid, pCGN3200 contains unique *Hind*III, *Not*I and *Kpn*I restriction sites at the 3'-end of the napin 3'-regulatory sequences as confirmed by sequence analysis.

The majority of the napin expression cassette is subcloned from pCGN3200 by digestion with *Hind*III and *Sac*I and ligation to *Hind*III and *Sac*I digested pIC19R (Marsh, et al. (1984) *Gene* 32:481-485) to make pCGN3212. The extreme 5' sequences of the napin promoter region are reconstructed by PCR using pCGN3200 as a template and two primers flanking the *Sac*I site and the junction of the napin 5'-promoter and the pUC backbone of pCGN3200 from the pCGN1808 construct. The forward primer contains *Cla*I, *Hind*III, 10 *Not*I, and *Kpn*I restriction sites as well as nucleotides 408-423 of the napin 5'-sequence (from the *Eco*RV site) and the reverse primer contains the complement to napin sequences 718-739 which include the unique *Sac*I site in the 5'-promoter. The PCR was performed using in a Perkin 15 Elmer/Cetus thermocycler according to manufacturer's specifications. The PCR fragment is subcloned as a blunt-ended fragment into pUC8 (Vieira and Messing (1982) *Gene* 19:259-268) digested with *Hinc*II to give pCGN3217. Sequenced of pCGN3217 across the napin insert verifies that 20 no improper nucleotides were introduced by PCR. The napin 5-sequences in pCGN3217 are ligated to the remainder of the napin expression cassette by digestion with *Cla*I and *Sac*I and ligation to pCGN3212 digested with *Cla*I and *Sac*I. The resulting expression cassette pCGN3221, is digested with 25 *Hind*III and the napin expression sequences are gel purified away and ligated to pIC20H (Marsh, *supra*) digested with *Hind*III. The final expression cassette is pCGN3223, which contains in an ampicillin resistant background, essentially identical 1.725 napin 5' and 1.265 3' regulatory sequences

as found in pCGN1808. The regulatory regions are flanked with *Hind*III, *Not*I and *Kpn*I restriction sites and unique *Sal*I, *Bgl*III, *Pst*I, and *Xho*I cloning sites are located between the 5' and 3' noncoding regions.

5       The 1200bp *Bal*I/*Pst*I thioesterase cDNA fragment described above is cloned into the napin expression cassette, pCGN3223, which has been digested with *Sal*I, and the *Sal*I site filled in using the Klenow fragment of DNA Polymerase I, followed by digestion with *Pst*I. The *Sal*I site is reconstituted in this ligation.

10      The napin/thioesterase/napin plasmid generated by these manipulations is digested with *Bam*HI and partially digested with *Kpn*I to generate an approximately 3.3kb fragment. This fragment contains ~1.7kb of napin 5' 15 noncoding sequence, the ~1200bp *Bal*I/*Pst*I thioesterase cDNA fragment and ~0.33kb of 3' napin noncoding region, the rest of the 1.265kb of the napin 3' having been deleted due to the *Bam*HI site in this region. The ~3.3kb fragment is ligated to *Kpn*I/*Bam*HI digested pCGN1557 or pCGN1578 20 (McBride and Summerfelt, *supra*) for plant transformation.

B.     A variety of methods have been developed to insert a DNA sequence of interest into the genome of a plant host to obtain the transcription or transcription and translation of the sequence to effect phenotypic changes.

25      Brassica Transformation

Seeds of *Brassica napus* cv. Westar are soaked in 95% ethanol for 2 min. surface sterilized in a 1.0% solution of sodium hypochlorite containing a drop of Tween 20 for 45 min., and rinsed three times in sterile, distilled water.

Seeds are then plated in Magenta boxes with 1/10th concentration of Murashige minimal organics medium (Gibco; Grand Island, NY) supplemented with pyridoxine (50 $\mu$ g/l), nicotinic acid (50 $\mu$ g/l), glycine (200 $\mu$ g/l), and 0.6%

- 5 Phytagar (Gibco) pH 5.8. Seeds are germinated in a Percival chamber at 22°C. in a 16 h photoperiod with cool fluorescent and red light of intensity approximately 65 $\mu$  Einsteins per square meter per second ( $\mu$ Em $^{-2}$ s $^{-1}$ ).

Hypocotyls are excised from 5-7 day old seedlings, cut  
10 into pieces approximately 4mm in length, and plated on feeder plates (Horsch et al., *Science* (1985) 227:1229-1231). Feeder plates are prepared one day before use by plating 1.0ml of a tobacco suspension culture onto a petri plate (100x25mm) containing about 30ml MS salt base  
15 (Carolina Biological, Burlington, NC) 100mg/l inositol, 1.3mg/l thiamine-HCl, 200mg KH<sub>2</sub>PO<sub>4</sub> with 3% sucrose, 2,4-D (1.0mg/l), 0.6% w/v Phytagar, and pH adjusted to 5.8 prior to autoclaving (MS 0/1/0 medium). A sterile filter paper disc (Whatman 3mm) is placed on top of the feeder layer  
20 prior to use. Tobacco suspension cultures are subcultured weekly by transfer of 10ml of culture into 100ml fresh MS medium as described for the feeder plates with 2,4-D (0.2mg/l), Kinetin (0.1mg/l). In experiments where feeder cells are not used hypocotyl explants are cut and placed  
25 onto a filter paper disc on top of MS0/1/0 medium. All hypocotyl explants are preincubated on feeder plates for 24 h. at 22°C in continuous light of intensity 30 $\mu$ Em $^{-2}$ s $^{-1}$  to 65 $\mu$ Em $^{-2}$ s $^{-1}$ .

Single colonies of *A. tumefaciens* strain EHA 101 containing a binary plasmid are transferred to 5ml MG/L broth and grown overnight at 30°C. Hypocotyl explants are immersed in 7-12ml MG/L broth with bacteria diluted to 5  $1 \times 10^8$  bacteria/ml and after 10-25 min. are placed onto feeder plates. Per liter MG/L broth contains 5g mannitol, 1g L-Glutamic acid or 1.15g sodium glutamate, 0.25g  $KH_2PO_4$ , 0.10g NaCl, 0.10g  $MGSO_4 \cdot 7H_2O$ , 1mg biotin, 5g tryptone, and 2.5g yeast extract, and the broth is adjusted to pH 7.0.

10 After 48 hours of co-incubation with *Agrobacterium*, the hypocotyl explants are transferred to B5 0/1/0 callus induction medium which contains filter sterilized carbenicillin (500mg/l, added after autoclaving) and kanamycin sulfate (Boehringer Mannheim; Indianapolis, IN)

15 at concentrations of 25mg/l.

After 3-7 days in culture at  $65\mu EM^{-2}s^{-1}$  continuous light, callus tissue is visible on the cut surface and the hypocotyl explants are transferred to shoot induction medium, B5BZ (B5 salts and vitamins supplemented with 3mg/l benzylaminopurine, 1mg/l zeatin, 1% sucrose, 0.6% Phytagar and pH adjusted to 5.8). This medium also contains carbenicillin (500mg/l) and kanamycin sulfate (25mg/l). Hypocotyl explants are subcultured onto fresh shoot induction medium every two weeks.

25 Shoots regenerate from the hypocotyl calli after one to three months. Green shoots at least 1cm tall are excised from the calli and placed on medium containing B5 salts and vitamins, 1% sucrose, carbenicillin (300mg/l), kanamycin sulfate (50mg/l) and 0.6% w/v Phytagar). After

2-4 weeks shoots which remain green are cut at the base and transferred to Magenta boxes containing root induction medium (B5 salts and vitamins, 1% sucrose, 2mg/l indolebutyric acid, 50mg/l kanamycin sulfate and 0.6% Phytagar). Green rooted shoots are tested for thioesterase activity.

Peanut Transformation

DNA sequences of interest may be introduced as expression cassettes, comprising at least a promoter 10 region, a gene of interest, and a termination region, into a plant genome via particle bombardment as described in European Patent Application 332 855 and in co-pending application USSN 07/225,332, filed July 27, 1988.

Briefly, tungsten or gold particles of a size ranging 15 from 0.5 $\mu$ M-3 $\mu$ M are coated with DNA of an expression cassette. This DNA may be in the form of an aqueous mixture or a dry DNA/particle precipitate.

Tissue used as the target for bombardment may be from 20 cotyledonary explants, shoot meristems, immature leaflets, or anthers.

The bombardment of the tissue with the DNA-coated particles is carried out using a Biolistics™ particle gun (Dupont; Wilmington, DE). The particles are placed in the barrel at variable distances ranging from 1cm-14cm from the 25 barrel mouth. The tissue to be bombarded is placed beneath the stopping plate; testing is performed on the tissue at distances up to 20cm. At the moment of discharge, the tissue is protected by a nylon net or a combination of nylon nets with mesh ranging from 10 $\mu$ M to 300 $\mu$ M.

Following bombardment, plants may be regenerated following the method of Atreya, et al., (*Plant Science Letters* (1984) 34:379-383). Briefly, embryo axis tissue or cotyledon segments are placed on MS medium (Murashige and 5 Skoog, *Physio. Plant.* (1962) 15:473) (MS plus 2.0 mg/l 6-benzyladenine (BA) for the cotyledon segments) and incubated in the dark for 1 week at 25 ± 2°C and are subsequently transferred to continuous cool white fluorescent light (6.8 W/m<sup>2</sup>). On the 10th day of culture, 10 the plantlets are transferred to pots containing sterile soil, are kept in the shade for 3-5 days and finally moved to greenhouse.

The putative transgenic shoots are rooted.

Integration of exogenous DNA into the plant genome may be 15 confirmed by various methods known to those skilled in the art.

#### Example 18 - Obtaining Other Plant Thioesterases

Having obtained sequence (amino acid and DNA) for Bay 20 thioesterase, similar genes from other plant sources can be readily isolated. In this example, two methods are described to isolate other thioesterase genes: (A) by DNA hybridization techniques using sequences or peptide sequence information from the Bay thioesterase gene and (B) 25 by immunological cross-reactivity using antibodies to the Bay protein as a probe.

In either of these techniques, cDNA or genomic libraries from the desired plants are required. Many methods of constructing cDNA or genomic libraries are

provided for example in Chapter 8 and 9 of Maniatis, et al. (*Molecular Cloning: A Laboratory Manual*, Second Edition (1989) Cold Spring Harbor Laboratory, Cold Spring Harbor, New York). The method described in Example 14 can also be  
5 used for cDNA library construction.

A. Probes for use in DNA hybridizations to isolate other plant thioesterase genes can be obtained from the Bay thioesterase gene sequences provided or alternatively by PCR using oligonucleotides from the Bay thioesterase  
10 peptide sequence provided.

In this example, the 800 bp PCR-generated DNA fragment is used as a probe. Northern analysis of embryo RNA from the desired plant species is conducted to determine appropriate hybridization conditions. RNA is isolated from  
15 embryo as described in Example 14.C., electrophoresed in a formaldehyde/agarose gel and transferred to a nylon membrane filter as described by Journey, et al. (*Focus* (1988) Bethesda Research Laboratories/Life Technologies, Inc., 10:5-7). The  $^{32}\text{P}$ -labeled probe (Random Primed DNA  
20 labeling kit, Boehringer Mannheim, Indianapolis, IN) is added to a hybridization solution containing 50% formamide, 6 x SSC (or 6 x SSPE), 5 x Denhardt's reagent, 0.5% SDS, and 100 $\mu\text{g}/\text{ml}$  denatured salmon sperm DNA fragments.

The hybridization solution containing the labeled  
25 probe is incubated with the Northern filter at approximately 40°C for 18 hours or longer to allow hybridization of the probe to homologous (50-80%) sequences. The filter is then washed at low stringency (room temperature to 42°C in about 1X SSC).

Hybridization and washing temperatures may be adjusted based on the estimated melting temperature of the probe as discussed in Beltz, et al. (*Methods in Enzymology* (1983) 100:266-285). In further testing the temperature is raised 5 either in the hybridization or washing steps, and/or salt content is lowered to improve detection of the specific hybridizing sequence.

A useful probe and appropriate hybridization and washing conditions having been identified as described 10 above, cDNA libraries are screened using the  $^{32}\text{P}$ -labeled fragment and optimized conditions.

For example, a camphor (*Cinnamomum camphora*) thioesterase clone may be isolated using nucleic acid and amino acid sequence information from the bay and safflower 15 thioesterase clones. Homology of the bay thioesterase cDNA clone to RNA isolated from developing camphor embryos is observed by Northern analysis as follows. Total RNA is isolated from 1g of developing camphor embryos by adaptation of the SDS/phenol extraction method described in 20 *Current Protocols in Molecular Biology*, pages 4.3.1-4.3.4 (Ausubel et al., eds. (1987); John Wiley & Sons). The grinding buffer for this extraction contains 100mM LiCl, 100mM Tris pH9, 10mM EDTA, 1%SDS and 0.5%  $\beta$ -mercaptoethanol. For extraction from 1g of embryos, 10ml 25 of grinding buffer plus 3ml of phenol equilibrated to pH8 are added to powdered embryos. The homogenization step may be conducted in a mortar instead of with a polytron, as described in the published method, and the heating step which follows homogenization in that method is omitted.

Centrifugation, phenol/chloroform extractions of the sample and LiCl precipitation of RNA are as described.

Total RNA (10-20 $\mu$ g) is electrophoresed in a formaldehyde/agarose gel and transferred to a nylon membrane filter as described by Journey et al. (supra). A probe for hybridization of the Northern filter is prepared from a SalI digest of pCGN3822, the full length bay thioesterase cDNA by PCR using oligonucleotides to the safflower thioesterase cDNA sequence to generate an approximately 1300bp fragment. The forward primer contains nucleotides 212 to 228 of the safflower thioesterase cDNA sequence (SEQ ID NO:38) and the reverse primer is the complement to nucleotides 1510-1526 of the cDNA sequence.

The fragment is gel purified using a Prep-A-Gene DNA purification kit (BioRad; Richmond, CA) and radiolabeled using a Boehringer Mannheim (Indianapolis, IN) random priming labeling kit. The Northern filter is hybridized overnight in 50% formamide, 5X SSC, 50mM sodium phosphate (pH7), 5X Denhardt's solution, 0.1% SDS, 5mM EDTA and 0.1mg/ml denatured DNA at 30°C. The filter is washed twice (15 minutes each wash) in 0.1X SSC, 0.1% SDS.

Autoradiography of the hybridized filter reveals a strong hybridization signal to an approximately 1300bp RNA band in the camphor embryo sample. This band is approximately the same size as the bay thioesterase mRNA.

To obtain a fragment of the camphor thioesterase gene, PCR is conducted using oligonucleotides to peptides conserved between the bay and safflower thioesterases. The forward primer, 828-1-2800, is a mixture of all possible

coding sequences for amino acids 101-107 of the bay thioesterase protein (SEQ ID NO:37) and amino acids 16-22 of safflower thioesterase peptide S828 (SEQ ID NO:26). These amino acid sequences are identical if one assumes the 5 unknown amino acid at position 18 of S828 is a serine. The primer (28bp) is designed such that an inosine (I) or cytosine (C) is incorporated where the base could be any of A, G, T or C. The unknown third base of the final amino acid, glycine, is not incorporated into the oligonucleotide 10 sequence, and the sequence CTGGATCC is added at the 5' end to include a *Bam*HI restriction site. The reverse primer, 829-2a-2798, is a mixture of all possible complements to the coding sequences for amino acids 271-276 of the bay thioesterase protein (SEQ ID NO:37) and amino acids 1-6 of 15 safflower thioesterase peptide S829 (SEQ ID NO:27). These 6 amino acid peptide regions differ only in their second amino acid (this residue having been identified as a glutamine in the bay and a lysine in the safflower peptides) and both amino acid codons are represented in the 20 oligonucleotide mixture. The reverse primer (26bp) incorporates an I or a C where the base could be A, G, T or C, and the sequence GCCTCGAG is added at the 5' end to add an *Xba*I restriction site.

Polymerase chain reactions are conducted using reverse 25 transcribed camphor RNA as template. The reactions are conducted in a Bioscycler Oven (Bios Corp.; New Haven, CT) programmed for the following cycles:

	N	P
	95°C for 2 min.	95°C for 15 sec.
	1 sec. drop to 65°C	1 sec. drop to 65°C
	hold 65°C for 1 sec.	hold 65°C for 1 sec.
	2 min. drop to 45°C	2 min. drop to 55°C
5	hold 45°C for 30 sec.	hold 55°C for 15 sec.
	1 sec. rise to 72°C	1 sec. rise to 72°C
	hold 72°C for 30 sec.	hold 72°C for 15 sec.
	1 sec. rise to 95°C	1 sec. rise to 95°C

10 Cycle N is run and repeated 6 times after which cycle P is run and repeated 37 times.

An approximately 500-600bp band is identified by agarose gel electrophoresis of the PCR products. This is the approximate fragment size predicted from analysis of 15 the distance between the peptides in the bay thioesterase sequence. The PCR fragment is subcloned into an appropriate cloning vector and its DNA sequence determined to verify thioesterase sequence. The fragment can then be utilized to screen a camphor cDNA or genomic library to 20 isolate a camphor thioesterase clone.

B. For immunological screening, antibodies to the Bay thioesterase are prepared by injecting rabbits or mice with the thioesterase protein purified from Bay or with the truncated thioesterase protein expressed in *E. coli* as 25 described Example 16.

Genes encoding related proteins are isolated by screening the cDNA library from the plant of interest that has been transferred to the expression vector lambda gt11, described in Chapter 12 of Maniatis, et al. (*supra*). The 30 libraries are then plated, induced to produce proteins from the cloned genes, and lifted onto membranes to immobilize for screening. The thioesterase antibodies are supplied to

the filters containing immobilized proteins to allow binding of the antibody to related proteins. Clones encoding thioesterase proteins are identified by detection of the antibody/protein complex on the nitrocellulose filters using a secondary antibody/enzyme conjugate system utilizing alkaline phosphate as described by Oberfelder (Focus (1989) BRL/Life Technologies, Inc. 11:1-5).

Analysis:

Clones identified using DNA hybridization or immunological screening techniques are then purified and the DNA isolated using techniques as provided in Maniatis, et al. (*supra*). DNA sequence of the genes is determined as described in Examples 14 and 15. In this manner, it is verified that the clones encode a related thioesterase.

Alternatively, the protein is expressed in *E. coli* as described above for the Bay thioesterase to show that it has the desired activity. The newly isolated plant thioesterase sequences can also be used to isolate genes for thioesterases from other plant species using the techniques described above.

By the above examples, demonstration of critical factors in the production of long-chain and medium-chain fatty acids is described. A protocol is provided to obtain partially purified C12-preferring acyl ACP thioesterase from the California Bay, various properties of the protein are described including methods to obtain and use amino acid and nucleic acid sequence related thereto. A partial cDNA sequence of the Bay thioesterase is also provided with

a demonstration of the activity of the polypeptide encoded thereby. A full sequence of the Bay thioesterase is also given with various constructs for use in host cells. In addition, methods to obtain a partially purified

5 preparation of a C10-preferring acyl-ACP thioesterase from *Cuphea hookeriana* is also provided. A long-chain preferring acyl-ACP thioesterase from safflower is also described. Through this invention, one can obtain the amino acid and nucleic acid sequences which encode plant

10 thioesterases from a variety of sources and for a variety of applications.

All publications and patent applications mentioned in this specification are indicative of the level of skill of

15 those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated

20 by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within

25 the scope of the appended claims.

## SEQUENCE LISTING

## 5 (1) GENERAL INFORMATION:

(i) APPLICANT: Davies, Huw Maelor  
Pollard, Michael Roman  
Voekler, Toni Alois  
10 Thompson, Gregory A

(ii) TITLE OF INVENTION: Plant Thioesterases  
15 (iii) NUMBER OF SEQUENCES: 40

## (iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Calgene, Inc.  
20 (B) STREET: 1920 Fifth Street  
(C) CITY: Davis  
25 (D) STATE: California  
(E) COUNTRY: USA  
(F) ZIP: 95616

## 30 (v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Diskette, 3.50 inch, 720 KB storage  
35 (B) COMPUTER: Apple Macintosh  
(C) OPERATING SYSTEM: Macintosh 6.0.7  
(D) SOFTWARE: MicrosoftWord 4.0

## 40 (vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: unassigned  
45 (B) FILING DATE:  
(C) CLASSIFICATION:

## (vii) PRIOR APPLICATION DATA:

50 (A) APPLICATION NUMBER: 07/662,007  
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55 (A) APPLICATION NUMBER: 07/514,030  
(B) FILING DATE: 26-APR-1990  
(A) APPLICATION NUMBER: 07/620,426  
60 (B) FILING DATE: 30-NOV-1990

## (viii) ATTORNEY/AGENT INFORMATION:

5 (A) NAME: Elizabeth Lassen

5 (B) REGISTRATION NUMBER: 31,845

10 (A) NAME: Donna E. Scherer

10 (B) REGISTRATION NUMBER: P-34,719

10 (C) REFERENCE/DOCKET NUMBER: CGNE 70-3 WO

## (ix) TELECOMMUNICATION INFORMATION:

15 (A) TELEPHONE: 916-753-6313

15 (B) TELEFAX: 916-753-1510

## 20 (2) INFORMATION FOR SEQ ID NO: 1:

## (i) SEQUENCE CHARACTERISTICS:

25 (A) LENGTH: 17 amino acids

25 (B) TYPE: amino acid

(D) TOPOLOGY: linear

30 (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

35 Try Pro Thr Trp Pro Asn Phe Val Leu Xaa Thr Met Leu Ile Gly Ala  
1 5 10 15

Gln

## 40 (2) INFORMATION FOR SEQ ID NO: 2:

## (i) SEQUENCE CHARACTERISTICS:

45 (A) LENGTH: 6 amino acids

45 (B) TYPE: amino acid

(D) TOPOLOGY: linear

50 (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

55 Asp Leu Met Trp Val Val  
1 5

## (2) INFORMATION FOR SEQ ID NO: 3:

60 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Xaa Gly Tyr Asn Pro Xaa Asp Ile Pro Phe Val Xaa  
10 1 5 10

(2) INFORMATION FOR SEQ ID NO: 4:

15 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 amino acids

20 (B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Thr Xaa Thr Leu Val Asp Val Val Pro Phe Val Ile Trp Phe Val Phe  
1 5 10 15

30 Ile Asp Asn Val Ala Val Lys  
20

(2) INFORMATION FOR SEQ ID NO: 5:

35 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16 amino acids

40 (B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Leu Asn Asp Xaa Thr Ala Asp Tyr Ile Gln Xaa Xaa Leu Thr Pro Arg  
1 5 10 15

50

(2) INFORMATION FOR SEQ ID NO: 6:

55 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15 amino acids

(B) TYPE: amino acid

60 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Ala Gly Gly Trp Val Phe Glu Thr Val Pro Asp Xaa Ile Phe Glu  
1 5 10 15

5

## (2) INFORMATION FOR SEQ ID NO: 7:

## (i) SEQUENCE CHARACTERISTICS:

10 (A) LENGTH: 17 amino acids

(B) TYPE: amino acid

15 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

20 Asn Glu Thr Gly Val Ile Xaa Val Val Met Xaa Val Ala Phe Gly Pro  
1 5 10 15

25 Ile

## (2) INFORMATION FOR SEQ ID NO: 8:

## (i) SEQUENCE CHARACTERISTICS:

30 (A) LENGTH: 17 amino acids

(B) TYPE: amino acid

35 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

40 Xaa Val Gly Ile Leu Gly Asp Gly Phe Gly Thr Thr Leu Glu Met Ser  
1 5 10 15

45 Lys

## (2) INFORMATION FOR SEQ ID NO: 9:

## (i) SEQUENCE CHARACTERISTICS:

50 (A) LENGTH: 10 amino acids

(B) TYPE: amino acid

55 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

60 Gly Ile Ser Val Ile Pro Ala Glu Pro Arg  
1 5 10

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

5 (A) LENGTH: 15 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

15 Leu Asn Asp Xaa Thr Ala Asp Tyr Ile Gln Gly Gly Leu Thr Pro  
1 5 10 15

(2) INFORMATION FOR SEQ ID NO: 11:

20 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 amino acids

25 (B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

Ser Val Gly Ile Leu Gly Asp Gly Phe Gly Thr Thr Leu Xaa Met Ser  
1 5 10 15

35 Lys

(2) INFORMATION FOR SEQ ID NO: 12:

40 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10 amino acids

45 (B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

50 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

Gly Ile Ser-Val Ile Pro Ala Glu Pro Arg  
1 5 10

55

(2) INFORMATION FOR SEQ ID NO: 13:

60 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 14 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(iii) MOLECULE TYPE: peptide

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

Tyr Val Ala Glu Val Phe Glu Thr Val Pro Asp Xaa Ile Phe  
1                   5                           10

10

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:

15

(A) LENGTH: 20 amino acids

(B) TYPE: amino acid

20

(D) TOPOLOGY: linear

(iii) MOLECULE TYPE: peptide

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

Ser Thr Asp Ile Leu Ala Val Met Asn Xaa Met Gln Phe Ala Thr Leu  
1                   5                           10                   15

30

Asn Xaa Ala Lys

20

(2) INFORMATION FOR SEQ ID NO: 15:

35

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 14 amino acids

(B) TYPE: amino acid

40

(D) TOPOLOGY: linear

(iii) MOLECULE TYPE: peptide

45

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

Xaa Xaa Ile Gly Pro Ala Phe Ile Asp Asn Val Ala Val Lys  
1                   5                           10

50

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:

55

(A) LENGTH: 14 amino acids

(B) TYPE: amino acid

60

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

Xaa Xaa Ile Gly Pro Ala Phe Ile Asp Asn Val Ala Val Lys  
1 5 10

5

## (2) INFORMATION FOR SEQ ID NO: 17:

## (i) SEQUENCE CHARACTERISTICS:

10 (A) LENGTH: 10 amino acids

(B) TYPE: amino acid

15 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

20 Ser Thr Ser Leu Ser Val Leu Met Asn Thr  
1 5 10

## 25 (2) INFORMATION FOR SEQ ID NO: 18:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6 amino acids

30 (B) TYPE: amino acid

(D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: peptide  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:Asp Ser Ile Phe Glu Ser  
1 5

40

## (2) INFORMATION FOR SEQ ID NO: 19:

## (i) SEQUENCE CHARACTERISTICS:

45 (A) LENGTH: 11 amino acids

(B) TYPE: amino acid

50 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

55 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:  
Asp Tyr Ile Gln Gly Gly Leu Thr Pro Xaa Trp  
1 5 10

60 (2) INFORMATION FOR SEQ ID NO: 20:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 amino acids

(B) TYPE: amino acid

5 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

Asp Ser Val Leu Xaa Ser Leu Thr Thr Val Xaa Gly Gly Ser Ser Glu  
1 5 10 15

Ala

15

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:

20 (A) LENGTH: 12 amino acids

(B) TYPE: amino acid

25 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

Asp Thr Val Xaa Val Glu Xaa Ile Ile Ala Asn Ser  
1 5 10

35 (2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:

40 (A) LENGTH: 14 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

45 (ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

50 Asp Xaa Phe Arg Gly Ile Ser Val Ile Pro Ala Glu Pro Arg  
1 5 10

(2) INFORMATION FOR SEQ ID NO: 23:

55 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 13 amino acids

(B) TYPE: amino acid

60 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

5 Asp Ser Phe Arg Gly Ile Ser Ile Val Ala Glu Pro Arg  
1 5 10

## (2) INFORMATION FOR SEQ ID NO: 24:

## 10 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10 amino acids

15 (B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

## 20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

Asp Trp Val Ile Glu Tyr Arg Pro Gly Val  
1 5 10

25

## (2) INFORMATION FOR SEQ ID NO: 25:

## (i) SEQUENCE CHARACTERISTICS:

30 (A) LENGTH: 15 amino acids

(B) TYPE: amino acid

35 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

40 Asp His Leu Leu Glu Leu Glu Gly Gly Ser Glu Val Leu Xaa Ala  
1 5 10 15

## 45 (2) INFORMATION FOR SEQ ID NO: 26:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27 amino acids

50 (B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

## 55 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

Gly Ser Leu Thr Glu Asp Gly Leu Ser Tyr Lys Glu Val Phe Ile Ile  
1 5 10 15

60

Arg Xaa Tyr Glu Val Gly Ile Asn Lys Thr Ala  
20 25

## (2) INFORMATION FOR SEQ ID NO: 27:

## (i) SEQUENCE CHARACTERISTICS:

5

(A) LENGTH: 37 amino acids

(B) TYPE: amino acid

10

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

Asn Lys His Val Asn Asn Val Thr Tyr Ile Gly Xaa Val Leu Glu Ser  
1 5 10 15

20

Ile Pro Gln Glu Val Ile Asp Thr His Glu Leu Gln Thr Ile Thr Leu  
20 25 30Asp Tyr Arg Arg Glu  
35

25

(2) INFORMATION FOR SEQ ID NO: 28:

## (i) SEQUENCE CHARACTERISTICS:

30

(A) LENGTH: 18 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

35

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

40

Ala Val Arg Thr Gly Glu Gln Pro Thr Gly Val Ala Val Gly Leu Lys  
1 5 10 15

Glu Ala

45

(2) INFORMATION FOR SEQ ID NO: 29:

## (i) SEQUENCE CHARACTERISTICS:

50

(A) LENGTH: 10 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

55

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

60

Lys Asp His Ala Ser Gly Gln Val Ile Gly  
1 5 10

## (2) INFORMATION FOR SEQ ID NO: 30:

## (i) SEQUENCE CHARACTERISTICS:

5 (A) LENGTH: 23 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

15 Asn Glu Asp Thr Arg Arg Leu Gln Lys Val Asn Asp Asp Val Glu Asp  
1 5 10 15Glu Tyr Leu Val Phe Ile Pro  
20

20

## (2) INFORMATION FOR SEQ ID NO: 31:

## (i) SEQUENCE CHARACTERISTICS:

25 (A) LENGTH: 9 amino acids

(B) TYPE: amino acid

30 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:  
His Ile Glu Ile Tyr Xaa Tyr Pro Ala  
1 5

## 40 (2) INFORMATION FOR SEQ ID NO: 32:

## (i) SEQUENCE CHARACTERISTICS:

45 (A) LENGTH: 28 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

50 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

55 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:  
CTGGATCCGA YATHYTNGCN GTNATGAA 28

## 60 (2) INFORMATION FOR SEQ ID NO: 33:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

5

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

GCCTCGAGCK NGGYTCNGCN GGRATNAC 28

15 (2) INFORMATION FOR SEQ ID NO: 34:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 210 base pairs

20

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

25 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34:

GAT ATT CTG GCC GTG ATG AAT CAC ATG CAG GAG GCT ACA CTT AAT CAT	48
Asp Ile Leu Ala Val MET Asn His MET Gln Glu Ala Thr Leu Asn His	
1 5 10 15	

GCG AAG AGT GTG GGA ATT CTA GGA GAT GGA TTC GGG ACG ACG CTA GAG	96
Ala Lys Ser Val Gly Ile Leu Gly Asp Gly Phe Gly Thr Thr Leu Glu	
20 25 30	

ATG AGT AAG AGA GAT CTG ATG TGG GTT GTG AGA CGC ACG CAT GTT GCT	144
MET Ser Lys Arg Asp Leu MET Trp Val Val Arg Arg Thr His Val Ala	
35 40 45	

GTG GAA CGG TAC CCT ACT TGG GGT GAT ACT GTA GAA GTA GAG TGC TGG	192
Val Glu Arg Tyr Pro Thr Trp Gly Asp Thr Val Glu Val Glu Cys Trp	
45 50 55 60	

GAA TGG TGC ATC TGG AAA	210
Glu Trp Cys Ile Trp Lys	
65 70	

50

(2) INFORMATION FOR SEQ ID NO: 35:

(i) SEQUENCE CHARACTERISTICS:

55

(A) LENGTH: 622 base pairs

(B) TYPE: nucleic acid

60 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 35:

5	ACG GCG GAT TAC ATA CAG GGA GGT TTG ACT CCT CGA TGG AAT GAT TTG Thr Ala Asp Tyr Ile Gln Gly Gly Leu Thr Pro Arg Trp Asn Asp Leu 1 5 10 15	48
10	GAT GTC AAT CAG CAT GTG AAC AAC CTC AAA TAC GTT GCC TGG GTT TTT Asp Val Asn Gln His Val Asn Asn Leu Lys Tyr Val Ala Trp Val Phe 20 25 30	96
15	GAG ACC GTC CCA GAC TCC ATC TTT GAG AGT CAT CAT ATT TCC AGC TTC Glu Thr Val Pro Asp Ser Ile Phe Glu Ser His His Ile Ser Ser Phe 35 40 45	144
20	ACT CTT GAA TAC AGG AGA GAG TGC ACG AGG GAT AGC GTG CTG CGG TCC Thr Leu Glu Tyr Arg Arg Glu Cys Thr Arg Asp Ser Val Leu Arg Ser 50 55 60	192
25	CTG ACC ACT GTC TCT GGT GGC TCG TCG GAG GCT GGG TTA GTG TGC GAT Leu Thr Thr Val Ser Gly Gly Ser Ser Glu Ala Gly Leu Val Cys Asp 65 70 75 80	240
30	CAC TTG CTC CAG CTT GAA GGT GGG TCT GAG GTA TTG AGG GCA AGA ACA His Leu Leu Gln Leu Glu Gly Ser Glu Val Leu Arg Ala Arg Thr 85 90 95	288
35	GAG TGG AGG CCT AAG CTT ACC GAT AGT TTC AGA GGG ATT AGT GTG ATA Glu Trp Arg Pro Lys Leu Thr Asp Ser Phe Arg Gly Ile Ser Val Ile 100 105 110	336
40	CCC GCA GAA CCG AGG GTG TAACTAATGA AAGAACATC TGTGAAAGTT Pro Ala Glu Pro Arg Val 115	384
45	TCTCCCATGC TGTTCGTGAG GATACTTTTT AGAACGCTGCA GTTGCATTG CTTGTGCAGA 444 ATCATGGTCT GTGGTTTAG ATGTATATAA AAAATAGTCC TGTAGTCATG AAACCTTAATA 504 TCAGAAAAAT AACTCAATGG GTCAAGGTAA TCGAAGTAGT CATTAAAGCT TTGAATATGT 564 TTTGTATTCC TCGGCTTAAT CTGTAAGCTC TTTCTCTTGC AATAAAAGTTC GCCTTTCG 622	

(2) INFORMATION FOR SEQ ID NO: 36:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 581 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: since

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

<sup>60</sup> 例如，2003年1月2日，烏拉圭總統胡安·馬丁內斯·烏爾塔斯（Juan Martínez Urtas）宣稱。

ATCAAATACT TGGCTGGATT TTTAAGAGCG TCCCAGACTA TATCTATGAG AATCATCATC 120  
5 TTTCTAGCAT CACTCTCGAA TACAGGAGAG AGTGCACAAG GGGCAGAGCA ACTGCAGTCC 180  
CTGACCAC TG TTTGTGGTGG CTCGTCCGAA GCTGGGGTCA TATGTGAGCA CCTACTCCAG 240  
CTTGAGGATG GGTCTGAGGT TTTGAGGGCA AGAACAGATT GGGAGGCCCA AGCGCACCGC 300  
10 ATAGTTCGA AGGCATTAGT GAGAGATTCC CGCAGCAAGA ACCGGCGTAA TTAATGACAG 360  
AAGCATCAGA TATA GTTTCT CCTGTGCTGT TCCTGAGAAT GCATCTTACA AGTCGTGGTT 420  
15 TGGATTGCTT GTGCAGAATC ATGGTTGTG CTTTCAGAAG TACATCTAAA TTAGTCCAAG 480  
TTATATGACT CCATATTGGA AAATAACTCG ATGAGTCGTG CTCTTGAAAT GGTCTTTAA 540  
GCTTGAAAT AAAGTACAC TTAATCCAAA AAAAAAAA A 581

20 (2) INFORMATION FOR SEQ ID NO: 37:

(i) SEQUENCE CHARACTERISTICS:

25 (A) LENGTH: 370 amino acids

(B) TYPE: amino acid

30 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 37:

35 Met Lys Ala Val Met Leu Ala Arg Asp Gly Arg Gly Met Lys Pro Arg  
1 5 10 15

Ser Ser Asp Leu Gln Leu Arg Ala Gly Asn Ala Pro Thr Ser Leu Lys  
20 25 30

40 Met Ile Asn Gly Thr Lys Phe Ser Tyr Thr Glu Ser Leu Lys Arg Leu  
35 40 45

Pro Asp Trp Ser Met Leu Phe Ala Val Ile Thr Thr Ile Phe Ser Ala  
45 50 55 60

Ala Glu Lys Gln Trp Thr Asn Leu Glu Trp Lys Pro Lys Pro Lys Leu  
65 70 75 80

50 Pro Gln Leu Leu Asp Asp His Phe Gly Leu His Gly Leu Val Phe Arg  
85 90 95

\* Arg Thr Phe Ala Ile Arg Ser Tyr Glu Val Gly Pro Asp Arg Ser Thr  
100 105 110

55 Ser Ile Leu Ala Val Met Asn His Met Gln Glu Ala Thr Leu Asn His  
115 120 125

Ala Lys Ser Val Gly Ile Leu Gly Asp Gly Phe Gly Thr Thr Leu Glu  
60 130 135 140

Met Ser Lys Arg Asp Leu Met Trp Val Val Arg Arg Thr His Val Ala  
145 150 155 160

242

	Val Glu Arg Tyr Pro Thr Trp Gly Asp Thr Val Glu Val Glu Cys Trp
	165 170 175
5	Ile Gly Ala Ser Gly Asn Asn Gly Met Arg Arg Asp Phe Leu Val Arg
	180 185 190
	Asp Cys Lys Thr Gly Glu Ile Leu Thr Arg Cys Thr Ser Leu Ser Val
10	195 200 205
	Leu Met Asn Thr Arg Thr Arg Arg Leu Ser Thr Ile Pro Asp Glu Val
	210 215 220
15	Arg Gly Glu Ile Gly Pro Ala Phe Ile Asp Asn Val Ala Val Lys Asp
	225 230 235 240
	Asp Glu Ile Lys Lys Leu Gln Lys Leu Asn Asp Ser Thr Ala Asp Tyr
	245 250 255
20	Ile Gln Gly Gly Leu Thr Pro Arg Trp Asn Asp Leu Asp Val Asn Gln
	260 265 270
	His Val Asn Asn Leu Lys Tyr Val Ala Trp Val Phe Glu Thr Val Pro
25	275 280 285
	Asp Ser Ile Phe Glu Ser His His Ile Ser Ser Phe Thr Leu Glu Tyr
	290 295 300
30	Arg Arg Glu Cys Thr Arg Asp Ser Val Leu Arg Ser Leu Thr Thr Val
	305 310 315 320
	Ser Gly Gly Ser Ser Glu Ala Gly Leu Val Cys Asp His Leu Leu Gln
	325 330 335
35	Leu Glu Gly Gly Ser Glu Val Leu Arg Ala Arg Thr Glu Trp Arg Pro
	340 345 350
	Lys Leu Thr Asp Ser Phe Arg Gly Ile Ser Val Ile Pro Ala Glu Pro
40	355 360 365
	Arg Val
	370
45	(2) INFORMATION FOR SEQ ID NO: 38:
	(i) SEQUENCE CHARACTERISTICS:
50	(A) LENGTH: 1561 base pairs
	(B) TYPE: nucleic acid
	(C) STRANDEDNESS: single
55	(D) TOPOLOGY: linear
	(ii) MOLECULE TYPE: cDNA to mRNA
60	(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 38:
	AGAGAGAGAG AGAGAGAGAG AGCTAAATTAA AAAAAGAAC CCAGAAGTGG GAAATCTTCC 60
	CCATGAAATA ACGGATCCTC TTGCTACTGC TACTACTACT ACTACAAACT GTAGCCATT 120

	ATATAATTCT ATATAATTT CAACRTGGCC ACCACCTCTT TAGCTTCCGC TTTCTGCTCG	180
5	ATGAAAGCTG TAATGTTGGC TCGTGATGGC CGGGGCATGA AACCCAGGAG CAGTGATTG	240
	CAGCTGAGGG CGGGAAATGC GCCAACCTCT TTGAAGATGA TCAATGGGAC CAAGTTCACT	300
	TACACGGAGA GCTTGAAAAG GTTGCCGTAC TGGAGCATGC TCTTTGCAGT GATCACAAACC	360
10	ATCTTTCCG CTGCTGAGAA GCAGTGGACC AATCTAGAGT GGAAGCCGAA GCCGAAGCTA	420
	CCCCAGTTGC TTGATGACCA TTTTGGACTG CATGGGTTAG TTTTCAGGCG CACCTTGCC	480
15	ATCAGATCTT ATGAGGTGGG ACCTGACCGC TCCACATCTA TACTGGCTGT TATGAATCAC	540
	ATGCAGGAGG CTACACTAA TCATGCGAAG AGTGTGGAA TTCTAGGAGA TGGATTCCGG	600
	ACGACGCTAG AGATGAGTAA GAGAGATCTG ATGTGGTTG TGAGACGCAC GCATGTTGCT	660
20	GTGGAACGGT ACCCTACTTG GGGTGATACT GTAGAAGTAG AGTGCTGGAT TGGTGCATCT	720
	GGAAATAATG GCATGCGACG TGATTTCTT GTCCGGACT GCAAAACAGG CGAAATTCTT	780
25	ACAAGATGTA CCAGCCTTTC GGTGCTGATG AATACAAGGA CAAGGAGGTT GTCCACAAATC	840
	CCTGACGAAG TTAGAGGGGA GATAGGGCCT GCATTCAATTG ATAATGTGGC TGTCAAGGAC	900
	GATGAAATTAA AGAAACTACA GAAGCTCAAT GACAGCACTG CAGATTACAT CCAAGGAGGT	960
30	TTGACTCCTC GATGGAATGA TTTGGATGTC AATCAGCATG TGAACAAACCT CAAATACGTT	1020
	GCCTGGTTT TTGAGACCGT CCCAGACTCC ATCTTGAGA GTCATCATAT TTCCAGCTTC	1080
35	ACTCTTGAAT ACAGGAGAGA GTGCACGAGG GATAGCGTGC TGCCTGCCCT GACCACTGTC	1140
	TCTGGTGGCT CGTCGGAGGC TGGGTTAGTG TGCGATCACT TGCTCCAGCT TGAAGGTGGG	1200
	TCTGAGGTAT TGAGGGCAAG AACAGAGTGG AGGCCTAACG TTACCGATAG TTTCAGAGGG	1260
40	ATTAGTGTGA TACCCGCAGA ACCGAGGGTG TAACTAATGA AAGAACATC TGTTGAAGTT	1320
	TCTCCCATGC TGTTCGTGAG GATACTTTT AGAACGCTGCA GTTTCATTG CTTGTGCAGA	1380
45	ATCATGGTCT GTGGTTTAG ATGTATATAA AAAATAGTCC TGTAGTCATG AAACCTTAATA	1440
	TCAGAAAAAT AACTCAATGG GTCAAGGTTA TCGAAGTAGT CATTAAAGCT TTGAAATATG	1500
	TTTTGTATTC CTCGGCTTAA TCTGTAAGCT CTTTCTCTTG CAATAAAGTT CGCCTTCAA	1560
50	T	1561

## (2) INFORMATION FOR SEQ ID NO: 39:

- 55 (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 14 amino acids
  - (B) TYPE: amino acid
  - 60 (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 39:

Leu Glu Trp Lys Pro Lys Pro Lys Leu Pro Glu Leu Leu Asp  
1 5 10

5

## (2) INFORMATION FOR SEQ ID NO: 40:

## (i) SEQUENCE CHARACTERISTICS:

10 (A) LENGTH: 28 amino acids

(B) TYPE: amino acid

15 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 40:

20 Leu Leu Asp Asp His Phe Gly Leu His Gly Leu Val Phe Arg Arg Thr  
1 5 10 15

Phe Ala Ile Arg Ser Tyr Glu Val Gly Pro Asp Arg  
20 25

25

What is claimed is:

1. A recombinant DNA construct comprising at least a portion of a plant thioesterase encoding sequence.
2. The construct of Claim 1 encoding a biologically active plant thioesterase.  
5
3. The construct of Claim 1 wherein said sequence is joined to a second nucleic acid sequence which is not naturally joined to said first sequence.
4. The construct of Claim 1 wherein said sequence  
10 is joined to a label.
5. The construct of Claim 1 wherein said sequence encodes a precursor plant thioesterase.
6. The construct of Claim 1 comprising, in the 5' to 3' direction of transcription, a transcriptional  
15 regulatory region functional in a host cell and said sequence.
7. The construct of Claim 6 wherein said sequence is a sense sequence.
8. The construct of Claim 6 wherein said sequence  
20 is an anti-sense sequence.
9. The construct of claim 6 further comprising, a translational regulatory region functional in a host cell immediately 5' to said sequence and a transcriptional/translational termination regulatory region 3' to said  
25 sequence.
10. The construct of Claim 1 comprising an expression cassette capable of producing a plant thioesterase in a host cell comprising, in the 5' to 3' direction of transcription, a transcriptional initiation

regulatory region functional in said host cell, a  
translational initiation regulatory region functional in  
said host cell, a DNA sense sequence encoding a  
biologically active plant thioesterase, and a  
5 transcriptional and translational termination regulatory  
region functional in said host cell, wherein said plant  
thioesterase encoding sequence is under the control of said  
regulatory regions.

11. The construct of Claim 10 wherein said host cell  
10 is a plant cell.

12. The construct of claim 11 wherein said  
transcriptional initiation region is obtained from a gene  
preferentially expressed in plant seed tissue.

13. The construct of Claim 1 wherein said plant  
15 thioesterase demonstrates preferential hydrolysis activity  
toward fatty acyl-acyl carrier protein (ACP) substrates.

14. The construct of Claim 1 wherein said plant  
thioesterase demonstrates preferential hydrolysis activity  
toward one or more medium-chain fatty acyl-carrier  
20 substrates.

15. The construct of Claim 1 wherein said sequence  
is obtainable from *Umbellularia californica* (Bay), *Cuphea*  
*hookeriana* (*Cuphea*) or *Carthamus tinctorius* (safflower).

16. A host cell comprising a plant thioesterase  
25 encoding sequence construct of any one of Claims 1-15.

17. The cell of claim 16 wherein said cell is a  
plant cell.

18. The cell of Claim 17 wherein said plant cell is  
*in vivo*.

19. The cell of Claim 17 wherein said plant cell is a *Brassica* plant cell.

20. A transgenic host cell comprising an expressed plant thioesterase.

5 21. The cell of Claim 20 wherein said host cell is a plant cell.

22. The cell of Claim 21 wherein said plant thioesterase is a medium-chain preferring acyl-carrier thioesterase.

10 23. The cell of Claim 22 wherein an untransformed plant cell is free of medium-chain preferring acyl-carrier thioesterase.

24. A method of producing a plant thioesterase in a host cell or progeny thereof comprising  
15 growing a host cell or progeny thereof comprising a construct of any one of Claims 10-12, under conditions which will permit the production of said plant thioesterase.

25. The method of Claim 24 wherein said host cell is  
20 a plant cell and said construct is integrated into the genome of said plant cell.

26. The method of Claim 25 wherein said plant cell is *in vivo*.

27. A host cell comprising a plant thioesterase  
25 produced according to Claim 24.

28. A cell of Claim 27 wherein said host cell is a plant host cell and said construct is integrated into the genome of said plant cell.

29. A plant thioesterase being substantially free from other plant proteins and having preferential hydrolase activity toward one or more medium-chain fatty acid acyl-carrier substrates.

5 30. The thioesterase of Claim 29 wherein said carrier is acyl carrier protein ("ACP").

31. The thioesterase of Claim 29 comprising a C12-preferring acyl-ACP thioesterase.

10 32. The thioesterase of Claim 29 comprising a C10-preferring acyl-ACP thioesterase.

33. The thioesterase of Claim 29 obtainable from an immature plant embryo.

15 34. The thioesterase of Claim 29 comprising a C12-preferring acyl-ACP thioesterase obtainable from *Umbellularia californica*.

35. The thioesterase of Claim 34 having a molecular weight of approximately 34 KDa.

36. The thioesterase of Claim 29 obtainable from *Cuphea*.

20 37. The thioesterase of Claim 36 comprising a C10-preferring acyl-ACP thioesterase obtainable from *Cuphea hookeriana*.

38. A method of producing a medium-chain free fatty acid comprising the steps of contacting, under enzyme reactive conditions, (1) a plant thioesterase having preferential hydrolase activity toward one or more medium-chain fatty acid acyl-carrier substrates and (2) a fatty acid acyl-carrier substrate capable of hydrolysis by said plant thioesterase, wherein said contacting is outside a

plant naturally associated with said thioesterase, and whereby a medium-chain fatty acid is released from said carrier.

39. The method of Claim 38 wherein said plant  
5 thioesterase is obtainable from *Umbellularia californica*.

40. The method of Claim 38 where said plant thioesterase is a *Umbellularia californica* C12-preferring acyl-ACP thioesterase.

41. The method of Claim 38 wherein said plant  
10 thioesterase is obtainable from *Cuphea hookeriana*.

42. The method of Claim 38 wherein said plant thioesterase is a *Cuphea hookeriana* C10-preferring acyl-ACP thioesterase.

43. The method of Claim 38 wherein said contacting  
15 is carried out *in vitro*.

44. A method of modifying the free fatty acids in a plant cell from a given percentage of longer chain fatty acids to shorter chain fatty acids comprising:

growing a plant cell having integrated in its genome a  
20 DNA construct, said construct comprising in the 5' to 3'  
direction of transcription, a transcriptional regulatory  
region functional in said plant cell and a plant  
thioesterase encoding sequence, under conditions which will  
permit the transcription of said plant thioesterase.

25 45. The method of Claim 44 wherein said plant  
thioesterase encoding sequence is an anti-sense sequence.

46. The method of Claim 44 wherein said construct  
further comprises a translational regulatory region  
functional in said plant cell immediately 5' to said plant

thioesterase encoding sequence and a transcriptional/translational termination regulatory region 3' to said sequence and wherein said plant thioesterase encoding sequence is a sense sequence.

5       47. The method of Claim 44 wherein said plant cell is an oilseed embryo plant cell.

48. The method of Claim 44 wherein the percentage of long chain free fatty acids is decreased and the percentage of medium-chain free fatty acids is increased.

10      49. A plant cell having a modified free fatty acid composition produced according to the method of any one of Claims 44-48.

50. A plant seed having a modified level of fatty acids as compared to a seed of said plant having a native 15 level of fatty acids produced according to a method comprising

growing a plant, having integrated into the genome of embryo cells a recombinant DNA sequence encoding a plant thioesterase under the control of regulatory 20 elements functional in seed during lipid accumulation, to produce seed under conditions which will promote the activity of said regulatory elements, and harvesting said seed.

51. The seed of Claim 50 wherein said plant is 25 *Brassica napus*.

52. The seed of Claim 50 wherein said seed is an oilseed.

53. A method of modifying the fatty acid composition of triglycerides produced from an oilseed crop plant from a

given percentage of longer chain fatty acids to shorter chain fatty acids comprising:

growing a plant cell having integrated in its genome a DNA construct, said construct comprising in the 5' to 3' direction of transcription, a transcriptional regulatory region functional in said plant cell and a plant thioesterase encoding sequence, under conditions which will permit the transcription of said plant thioesterase.

54. The method of Claim 53 wherein said plant thioesterase encoding sequence is an anti-sense sequence.

55. The method of Claim 53 wherein said construct further comprises a translational regulatory region functional in said plant cell immediately 5' to said plant thioesterase encoding sequence and a transcriptional/translational termination regulatory region 3' to said sequence and wherein said plant thioesterase encoding sequence is a sense sequence.

56. The method of Claim 53 wherein said plant cell is an oilseed embryo plant cell.

20 57. The method of Claim 53 wherein the percentage of long chain fatty acids is decreased and the percentage of medium-chain fatty acids is increased.

58. A plant cell having a modified fatty acid composition of triglycerides produced according to the method of any one of Claims 53-57.

59. The method of any one of Claims 53 to 57 wherein said crop plant is selected from the group consisting of rapeseed, sunflower, safflower, cotton, cuphea, soybean, peanut, coconut, oil palm and corn.

60. A plant seed oil of a plant having an endogenous proportion of longer chain fatty acids to shorter chain fatty acids comprising a plant seed oil having a modified proportion of longer chain fatty acids to shorter chain fatty acids.

61. The oil of Claim 60 comprising a *Brassica napus* oil.

62. A plant seed oil separated from a seed produced according to any one of Claims 50-52.

63. A triglyceride oil from a plant selected from the group consisting of rapeseed, sunflower, cotton, cuphea, soybean, peanut, coconut, oil palm and corn wherein the fatty acid chain length composition of the oil has been modified from a given percentage of longer chain fatty acids to shorter chain fatty acids by a method comprising:

growing a plant cell have integrated in its genome a DNA construct, said construct comprising in the 5' to 3' direction of transcription, a transcriptional regulatory region functional in said plant cell and a plant thioesterase encoding sequence, under conditions which will permit the transcription of said plant thioesterase.

64. The oil of Claim 63 wherein said plant thioesterase encoding sequence is an anti-sense sequence.

65. The oil of Claim 64 wherein said construct further comprises a translational regulatory region functional in said plant cell immediately 5' to said plant thioesterase encoding sequence and a transcriptional/translational termination regulatory region

3' to said sequence and wherein said plant thioesterase encoding sequence is a sense sequence.

66. The oil of Claim 65 wherein the percentage of long chain fatty acids is decreased and the percentage of 5 medium-chain free fatty acids is increased.

Peptide 701  
SerThrAspIleLeuAlaValMetAsnXxxMetGln...

..BamHI  
ctggatccgacataCTTGCTATGAA 3' ----->  
T C T C C C  
T

Peptide 698  
GlyIleSerValIleProAlaGluProArg

XhoI  
<-----3' CAITAAGGICCGICCTCGGIGCqaagctccg  
C G C C T C T  
T

FIGURE 1

<-- PCR 5' GATATTCTGGCGTGTGATCACATGCAGGGCTACACTTAATCATGCGAAGAGTGTGGAAATTCTA  
 AspIleLeuAlaValMetAsnHisMetGlnGluAlaThrLeuAsnHisSerValGlyIleLeu  
 -----|-----  
 101-----Xxx-----Phe-----  
 -----|-----  
 EcoRI |  
 -----|-----  
 69 9  
 GGGAGATGGATTGGGACGACCGCTAGAGATGGAGTAAGGAGAGATCTGATGTGGGTGTGAGACGCACGCCAT  
 GlyAspGlyPheGlyThrThrLeuGluMETSerLysArgAspLeuMETHTrpValValArgArgThrHis  
 -----|-----  
 768-----  
 -----|-----  
 BgIII |  
 -----|-----  
 697-----Xxx-----  
 -----|-----  
 KpnI |  
 -----|-----  
 768-----  
 GTTGCTGTGGAACCGGTACCCtACTTGGGGTGTACTGTAGAAAGTAGCTGGGAATGGTGCATCTGG  
 ValAlaValGluArgTyrProThrTrpGlyAspThrValGluValGluCysTrpGluTrpCysIleTrp  
 -----|-----  
 212 213

FIGURE 2  
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ACGGCGATTACATACAGGGAGGTTTGAECTCCCTCGATGGAATGATTGGATGTCAATCAGGCATGTGAAC (69)  
ThrAlaAspTyrIleGlnGlyLeuThrProArgTrpAsnAspLeuAspValAsnGlnHisValAsn  
-----696-----

AACCTCAAATAACGTTGGCTGGGTTTGGAGACCGTCCCAGACTCCATCTTTGAGAGTCATCATATTCC (138)  
AsnLeuLysTyrValAlaTrpValPheGluThrValProAspSerIlePheGluSerHisHisIleSer  
-----699-----  
-----XXX-----

AGCTTCACTCTTGAATAACAGGAGAGAAGTGCACGGGATAGCGGTGCTGCGGTCCCTGACCACTGTCTCT (207)  
SerPheThrLeuGlutYrArgArgGluCysThrArgAspSerValLeuArgSerLeuThrThrValSer  
-----767-----  
-----XXX-----

FIGURE 2  
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<-- lib. 5' GGTGGCTCGTGGAGGCTGGTTAGTGTGGATCACTGCTCCAGCTTGAGGTGGTCTGAGGTATTG (276)  
 GlyGlySerSerGluAlaGlyLeuValCysAspHisLeuGlnLeuGluGlyGlySerGluValLeu  
 -----  
 Glu-----773-----

HindIII  
 |  
 AGGGCAAGAACAGAGTGGAGGCCTAACGCTTACCGATAAGCTTAGTTCAGAGGGATTAGTGTGATAACCCGGAGAA (345)  
 ArgAlaArgThrGluTrpArgProLysLeuThrAspSerPheArgGlyIleSerValLeuProAlaGlu  
 -----698-----

--> PCR 3'  
 CCGAGGGGTGTAACTAATGAAGAACATCTGGTTGAAGTTCTCCCATGGCTGTTGAGGATACTTTT (414)  
 ProArgVal .  
 -----

**FIGURE 2**  
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$\text{PstI}$   AGAAGCTGCAGTTGCATTGCTTGTGCAGAACATGGTCTGTTAGATGTATAAAAAATAGTC	(483)
CTGTAGTCATGAAACTTAATCAGAAAAATAACTCAATGGGTCAAGGTATCGAAGTAGTCATTAAAG	(552)
CTTTGAATATGTTTGTATTCCCTCGGCTTAATCTGTAAGCTCTCTGCATAAAAGTTCGCCTTTCG	(622)
lib 3' -->	

**FIGURE 2**  
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## **SUBSTITUTE SHEET**

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10	20	30	40	50	60	70
GGATTACATACAGGGAGGTTTGA	CTCGATGGAATGATTGGATGTC	AAATCAGGCTTACGCTCCAG	CTGCAATCAGGCATGTGA	AAACACCTC		
X	10	20	30	40	50	60
80	90	100	110	120	130	140
AAATACGTTGCC	TGGGTTTGAGACCGTCCCAG	ACTCCATCTTGAGAGTCAT	CATATTCCAGCTTCA			
X	70	80	90	100	110	120
150	160	170	180	190	200	210
CTCTTGAA	TACAGGAGAGGTGCACGGG	GATAGGGTCACTGCTGAC	CTGCGGCTCCAGCTTGAG	GGTCTGAGCTG		
X	140	150	160	170	180	190
220	230	240	250	260	270	280
CGTCGGAGG	GCTGGTTAGTGTGCGATCA	CTTGCTCCAGCTTGAGGT	ATGTTGAGGTCTGAGG	TATTGAGGGCAAG		
X	210	220	230	240	250	260
290	300	310	320	330	340	
AACAGAGTGG	-AGGCCTAACGTTACCG-	ATAGTTCA	GAGGGATTAGTGT--	GATA	CCCCGAG	
X	280	290	300	310	320	330
AACAGAT	TTGGGAGGCCAAGGGCACCG	CATAGTTG	GAGAGATTAGT	GAGAGAT	TCCCGCAGAAC	

**FIGURE 3**

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360      370      380      390      400      410  
 GGGTGTAACTAATGAAAGCATCTGTTGAAGTTCTCCCCATGCTGTCGTGAGGGATAACTTTAGAAG  
 ||| | | | | | | | | | | | | | | | | | | | | | | | | |  
 CGCGTAATTAAATGACAGAAGCATCAGATATAGTTCTCCTGCTGTTGAGAATGCATCTTACAAG  
 350      360      370      380      390      400      410  
  
 430      440      450      460      470      480  
 CTGCAGTTGCATTGCTTGTGCAGAATCATGGTCTGTGGTTTAGATGTTATAAAAATAGTCCCTGTAG  
 ||| | | | | | | | | | | | | | | | | | | | | | | | |  
 TCGTGGTTGGATTGCTTGCAGAAATCATGGTTGTGCTTTCAAGGTACATCTAAATTAGTCCA--AG  
 420      430      440      450      460      470      480  
  
 500      510      520      530      540      550  
 TCATGAAACTTAATATCAGAAAAATAACTCAAATGGGTCAGGTTATC--GAAGTAGTCATTAAAGCTTIG  
 ||| | | | | | | | | | | | | | | | | | | | | | | | | |  
 TTATATGACTCCATATTGAAAA-TAACTCGATGAGTC---GTGCTCTGAAATGGTCTTTAAAGCTTIG  
 490      500      510      520      530      540  
  
 560      570      580      590      600  
 AATATGTTTGTATTCCCTCGGCTTAATCTGTAAGCTCTTC  
 ||| | | | | | | | | | | | | | | | | | | | | | | | | |  
 AAA-----TAAAGTACCACTTAATCCAAAAAA  
 550      560      570      580

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**FIGURE 3**  
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Met Lys Ala Val Met Leu Ala Arg Asp	Gly Arg Gly Met Lys Pro Arg
1 5	10 15
Ser Ser Asp Leu Gln Leu Arg Ala Gly Asn Ala Pro Thr Ser Leu Lys	
20 25	30
Met Ile Asn Gly Thr Lys Phe Ser Tyr Thr Glu Ser Leu Lys Arg Leu	
35 40	45
Pro Asp Trp Ser Met Leu Phe Ala Val Ile Thr Thr Ile Phe Ser Ala	
50 55	60
Ala Glu Lys Gln Trp Thr Asn Leu Glu Trp Lys Pro Lys Pro Lys Leu	
65 70	75 80
Pro Gln Leu Leu Asp Asp His Phe Gly Leu His Gly Leu Val Phe Arg	
85 90	95
Arg Thr Phe Ala Ile Arg Ser Tyr Glu Val Gly Pro Asp Arg Ser Thr	
100 105	110
Ser Ile Leu Ala Val Met Asn His Met Gln Glu Ala Thr Leu Asn His	
115 120	125
Ala Lys Ser Val Gly Ile Leu Gly Asp Gly Phe Gly Thr Thr Leu Glu	
130 135	140
Met Ser Lys Arg Asp Leu Met Trp Val Val Arg Arg Thr His Val Ala	
145 150	155 160

**FIGURE 4A**  
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Val	Glu	Arg	Tyr	Pro	Thr	Trp	Gly	Asp	Thr	Val	Glu	Val	Cys	Trp	
				165					170				175		
Ile	Gly	Ala	Ser	Gly	Asn	Asn	Gly	Met	Arg	Arg	Asp	Phe	Leu	Val	Arg
				180				185				190			
Asp	Cys	Lys	Thr	Gly	Glu	Ile	Leu	Thr	Arg	Cys	Thr	Ser	Leu	Ser	Val
				195			200				205				
Leu	Met	Asn	Thr	Arg	Thr	Arg	Arg	Leu	Ser	Thr	Ile	Pro	Asp	Glu	Val
				210			215				220				
Arg	Gly	Glu	Ile	Gly	Pro	Ala	Phe	Ile	Asp	Asn	Val	Ala	Val	Lys	Asp
				225			230				235				240
Asp	Glu	Ile	Lys	Lys	Leu	Gln	Lys	Leu	Asn	Asp	Ser	Thr	Ala	Asp	Tyr
				245					250				255		
Ile	Gln	Gly	Leu	Thr	Pro	Arg	Trp	Asn	Asp	Leu	Asp	Val	Asn	Gln	
			260				265					270			
His	Val	Asn	Leu	Lys	Tyr	Val	Ala	Trp	Val	Phe	Glu	Thr	Val	Pro	
				275					280				285		
Asp	Ser	Ile	Phe	Glu	Ser	His	His	Ile	Ser	Ser	Phe	Thr	Leu	Glu	Tyr
			290				295				300				

**SUBSTITUTE SHEET****FIGURE 4A  
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Arg Arg Glu Cys Thr Arg Asp Ser Val Leu Arg Ser Leu Thr Thr Val  
305 310 315 320  
Ser Gly Gly Ser Ser Glu Ala Gly Leu Val Cys Asp His Leu Leu Gln  
325 330 335  
Leu Glu Gly Gly Ser Glu Val Leu Arg Ala Arg Thr Glu Trp Arg Pro  
340 345 350  
Lys Leu Thr Asp Ser Phe Arg Gly Ile Ser Val Ile Pro Ala Glu Pro  
355 360 365  
Arg Val  
370

**FIGURE 4A**  
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AGAGAGAGAG	AGAGAGAGAG	AGCTAAATTAA	AAAAAAAAC	CCAGAAGTGG	GAAATCTCC	60
CCATGAAATA	ACGGATCCTC	T TGCTACTGC	TACTACTACT	ACTACAAACT	GTAGCCATT	120
ATATAATTCT	ATATAATTCTT	CAACRTGGCC	ACCACCTCTT	TAGCTTCGC	TTTCTGCTCG	180
ATGAAAGCTG	TAATGTGGC	TCGTGATGGC	CGGGGCATGA	AACCCAGGAG	CAGTGATTG	240
CAGCTGAGGG	CGGAAATGC	GCCAACCTCT	TGAAAGATGA	TCAATGGAC	CAAGTTCACT	300
TACACGGAGA	GCTTGAAAG	GTTGCCTGAC	TGGACCATGC	TCTTTGCAGT	GATCACAAACC	360
ATCTTTTCGG	CTGCTGAGAA	GCAGTGGACCC	ATCTAGAGT	GAAGGCCAA	GCCGAAAGCTA	420
CCCCAGTTGC	TTGATGACCA	TTTGGACTG	CATGGGTAG	TTTTCAGGCG	CACCTTGCC	480
ATCAGATCTT	ATGAGGTGGG	ACCTGACCGC	TCCACATCTA	TACTGGCTGT	TATGAATCAC	540
ATGCAGGAGG	CTACACTTAA	TCATGCCAAG	AGTGTGGAA	TTCTAGGAGA	TGGATTCCGG	600
ACGACGGCTAG	AGATGAGTAA	GAGAGATCTG	ATGTTGGTTG	TGAGACCCAC	GCATGTTGCT	660
GTGGAACGGT	ACCCTACTTG	GGGTGATACT	GTAGAAGTAG	AGTGCTGGAT	TGGTGCATCT	720
GGAATAATG	GCATGCCAG	TGATTTCCTT	GTCCGGACT	GCAAAACAGG	CGAAATTCTT	780
ACAAGATGTA	CGAGCCTTTC	GGTGTGATG	AATACAGGAA	CAAGGAGGTT	GTCCACAAATC	840

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**FIGURE 4B**  
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CCTGACGAAG	TAGAGGGAA	GATAGGGCCT	GCATTCAATTG	ATAATGTGGC	TGTCAAGGAC	900
GATGAAATTAA	AGAAAACATACA	GAAGCTCAAAT	GACAGCACTG	CAGATTACAT	CCAAGGAGGT	960
TTGACTCCCTC	GATGGAATGAA	TTTGGATGTC	ATTCAGGATG	TGAACAAACCT	CAAATACGTT	1020
GCCTGGGTTT	TTGAGACCGT	CCCAGACTCC	ATCTTGAGA	GTCATCATAT	TTCCAGCTTC	1080
ACTCTTGAAT	ACAGGAGAGA	GTGCCACGAGG	GATAGCCTGTC	TGGGGTCCCT	GACCACTGTC	1140
TCTGGTGGCT	CGTCCGGAGGC	TGGGTTAGTG	TGCGATCACT	TGCTCCAGCT	TGAAGGTGGG	1200
TCTGAGGTAT	TGAGGGCAAG	AACAGAGGTGG	AGGCCTAAGC	TTACCGATAG	TTTCAGAGGG	1260
ATTAGTGTGA	TACCCCGCAGA	ACCGAGGGTG	TAACATAATGA	AAGAACATC	TGTTGAAGTT	1320
TCTCCCCATGC	TGTTCGTGAG	GATACTTTT	AGAAGCTGCA	GTTTGCATTG	CTTGTGCAGA	1380
ATCATGGTCT	GTGGTTTAG	ATGTATATAA	AAAATAGTCC	TGTAGTCATG	AAACTTAATA	1440
TCAGAAAAAT	AACTCAATGG	GTCAAGGTTA	TGGAAGTAACT	CATTAAAGCT	TTGAAATATG	1500
TTTTGTATTC	CTCGGGCTTAA	TCTGTAAGCT	CTTTCTCTTG	CAATAAAGTT	CGCCTTTCAA	1560
				T		1561

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**FIGURE 4B**  
**2 / 2**

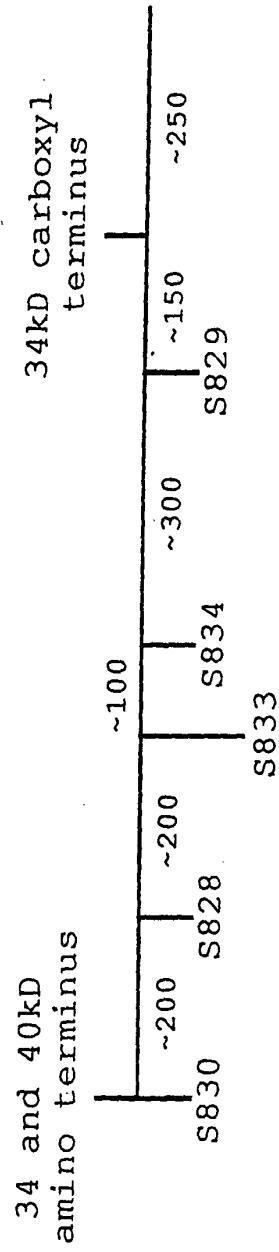


FIGURE 5

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